PURIFICATION AND CHARACTERISATION 0F MYELOID BLAST CELLS FROM HUMAN FETAL LIVER AND STUDIES OF CHANGES IN INOSITOL METABOLISM DURING THEIR DIFFERENTIATION TOWARDS MONOCYTES.

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ABSTRACT

PURIFICATION AND CHARACTERISATION OF MYELOID BLAST CELLS FROM HUMAN FETAL LIVER AND STUDIES OF CHANGES IN INOSITOL METABOLISM DURING THEIR DIFFERENTIATION TOWARDS MONOCYTES.

A homogeneous population of myeloid blast cells was purified by negative selection from human fetal liver by enzyme digestion of liver tissue, ficoll fractionation, indirect erythrocyte rosette sedimentation of unwanted cells, after coating these cells with monoclonal antibodies directed against erythroblasts and macrophages, and finally by cell elutriation. Characterisation of these cells confirmed their undifferentiated status and in culture these cells generated only neutrophils and macrophages. Treatment of these cells with 10nM phorbol myristate acetate (PMA) induced rapid terminal monocyte differentiation of 62% of this population and also completely inhibited the neutrophil differentiation of the remaining cells. The undifferentiated blast cells were maintained in culture in a serum-free medium containing 100 U/ml interleukin-3 and 1mg/l inositol which permitted the equilibrium labelling of cells with [^{3}H] myo-inositol and the subsequent analysis of concentrations of inositol metabolites within these cells. High concentrations of various inositol metabolites, similar to those found in HL60 cells, were observed in normal myeloid blast cells and following PMA-induced monocyte differentiation of these cells significant changes occurred - namely a decrease in inositol tetrakisphosphate ($InsP_4$) and inositol pentakisphosphate ($InsP_5$) and an increase in glycerophospho-inositol (GPI). These changes in response to PMA, with the exception of the rise in GPI, are similar to those reported in HL60 cells undergoing monocyte differentiation and suggest that abundant inositol polyphosphates may play an important role in myeloid differentiation.

A second section of this thesis describes a case of primary myelofibrosis associated with defective erythropoiesis in which cytogenetic analysis suggested the possible involvement of a new putative human oncogene regulating erythropoiesis. Subsequent analysis using a cDNA probe to this putative oncogene (pSEA, the human homologue of the viral oncogene SEA of the S13 avian erythroblastosis virus) indicated that this locus was not disrupted by the chromosomal translocation observed in this patient.

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LIST OF ABBREVIATIONS

61D3	monoclonal antibody directed against monocytes/ macrophages
ACTH	adrenocorticotrophic hormone
AGF4.48	monoclonal antibody directed against granulocyte specific antigen (CD15)
ANAE	alpha naphthyl acetate esterase
BFU-E	blast forming unit - erythroid
BK19.45	monoclonal antibody directed against common leucocyte antigen (CD45)
BSA	bovine serum albumin
CAE	chloroacetate esterase
CFCs	colony forming cells
CFU-E	colony forming unit - erythroid
CFU-GM	colony forming unit - granulocyte,monocyte
CFU-Meg	colony forming unit - megakaryocyte
CFU-S	colony forming unit - spleen
CSFs	colony-stimulating factors
DAG	1,2 - diacylglycerol
DPM	disintegrations per minute
EPO	erythropoietin
FCS	fetal calf serum
FDCP	factor dependent continuous cell lines, Paterson laboratories
GPI	glycerophospho-inositol
HPLC	high performance liquid chromatography
[^{3}H]TdR	tritiated thymidine
IL	interleukin ; numbered 1 - 7,9,11
InsP	inositol monophosphate
$InsP_2$	inositol biphosphate
$InsP_3$	inositol triphosphate
$InsP_4$	inositol tetrakisphosphate
$InsP_5$	inositol pentakisphosphate
$InsP_6$	inositol hexakisphosphate
LTBMC	long term bone marrow culture
MGG	May-Grunwald-Giemsa
rh	recombinant human
SEM	Standard error of mean

PAS	periodic acid-Schiff
PBMNC	peripheral blood mononuclear cells
PDGF	platelet derived growth factor
PHA - LCM	phytohaemagglutinin stimulated leucocyte conditioned medium
PHSCs	pluripotential haemopoietic stem cells
PIP_2	phospho-inositol (4,5) bisphosphate
PMA	phorbol myristate acetate
PMF	primary myelofibrosis
Ptd Ins	phosphatidyl-inositol
Ptd Ins(4,5)P_2	phosphatidyl-inositol (4,5) biphosphate
Ret40F	monoclonal antibody directed against glycophorin C
SCF	stem cell factor (c-kit ligand)
SCID	severe combined immunodeficiency
TNF	tumour necrosis factor

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INTRODUCTION CHAPTER 1.
HAEMOPOIESIS AS A MODEL SYSTEM IN DEVELOPMENTAL BIOLOGY

The human haemopoietic system generates and maintains throughout adult life at least eight different terminally differentiated and functional cell types which are derived from a finite number of pluripotential stem cells (Figure 1). This capacity to generate and maintain populations of widely diverse functional cell types from a limited number of ancestral cells is an important property of successful embryonic and evolutionary development which is common to all higher organisms. The sequence of events, whereby stem cells give rise to diverse functional end cells involves the cellular processes of self-renewal, lineage commitment, proliferation and differentiation which are extremely complex, and difficult to study. However, an understanding of these events at the molecular level is obviously important in developmental biology and in resolving the perturbations in these processes which occur in disease states such as lineage dysplasias and neoplasias. The haemopoietic system is particularly well suited to studies of development as it is possible to access sources of pluripotential human haemopoietic stem cells and primitive progenitor cells from tissues such as adult bone marrow, fetal liver and neonatal cord blood. The availability of these cells has permitted the development of in vitro model systems to study important events which regulate haemopoiesis.

The human haemopoietic system is subject to perturbation of the cellular processes which control lineage commitment, proliferation and differentiation resulting in disease states such as lineage aplasias, lineage dysplasias and lineage neoplasias. Cells isolated from patients with these disorders can also be subjected to in vitro study and knowledge of the biochemical and molecular events associated with these disease states may lead to new ways of treating and preventing these conditions and to a new understanding of the events which govern normal developmental processes. Therefore, study of the developmental processes of both normal and malignant haemopoietic cell populations has the potential to identify the biochemical and molecular events which regulate normal cell development and the mechanism by which they are perturbed in malignancy.

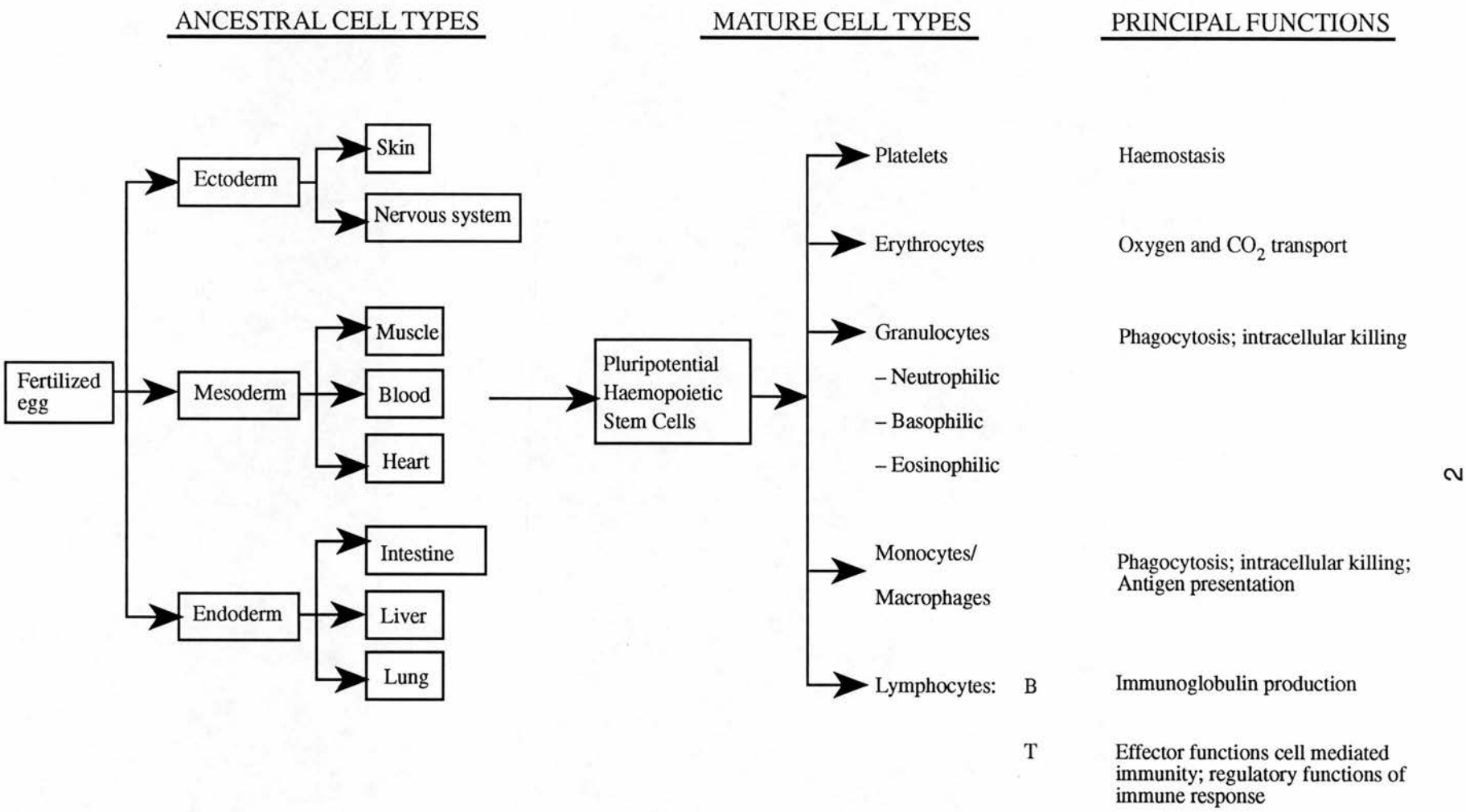

Figure 1: Origin of terminally differentiated cell types within the human haemopoietic system and their principal functions.

CHAPTER 2.
STRUCTURE AND REGULATION OF THE HAEMOPOIETIC SYSTEM

Over the past 25 years considerable advances have been made in our knowledge of the structure and regulation of the haemopoietic system. Evidence supports the existence of, as yet unpurified, pluripotential haemopoietic stem cells (PHSCs) which are capable of generating and maintaining throughout adult life eight different terminally differentiated and functional cell types (Figure 1). Stem cells exhibit properties of self-renewal and pluripotency. PHSCs give rise to intermediate precursor cells, called progenitors or colony forming cells (CFCs), which were first recognised by their ability to generate colonies of differentiated progeny in response to appropriate colony-stimulating factors (CSFs). These progenitor cells are transit amplifying populations which exhibit properties of limited self-renewal capacity, commitment to a restricted range of lineages and both proliferation and differentiation in response to appropriate CSFs. Progenitor cells eventually become restricted to a single lineage and ultimately generate functional mature cells that are terminally differentiated and which have no further potential for self-renewal (Figure 2). Such terminally differentiated cells have a finite life span and normal numbers of peripheral blood cells are maintained by a balanced continual production of new cells. The mechanisms whereby normal haemopoiesis is regulated are outlined in more detail below.

2.1 Pluripotential haemopoietic stem cells

2.1.1. Evidence for the existence of PHSCs

The first experimental evidence for the existence of pluripotential haemopoietic stem cells (PHSCs) came from reconstitution experiments in mice, where bone marrow cells harvested from normal mice were used to reconstitute the lymphohaemopoietic system of mice that had received lethal doses of irradiation (Ford et al, 1956; Micklem et al, 1966). When low numbers of marrow cells were injected, colonies containing megakaryocytes, granulocytes and erythroid cells but not lymphoid cells could be identified in the spleens of irradiated mice (Till and McCulloch, 1961). These colonies were found to have been

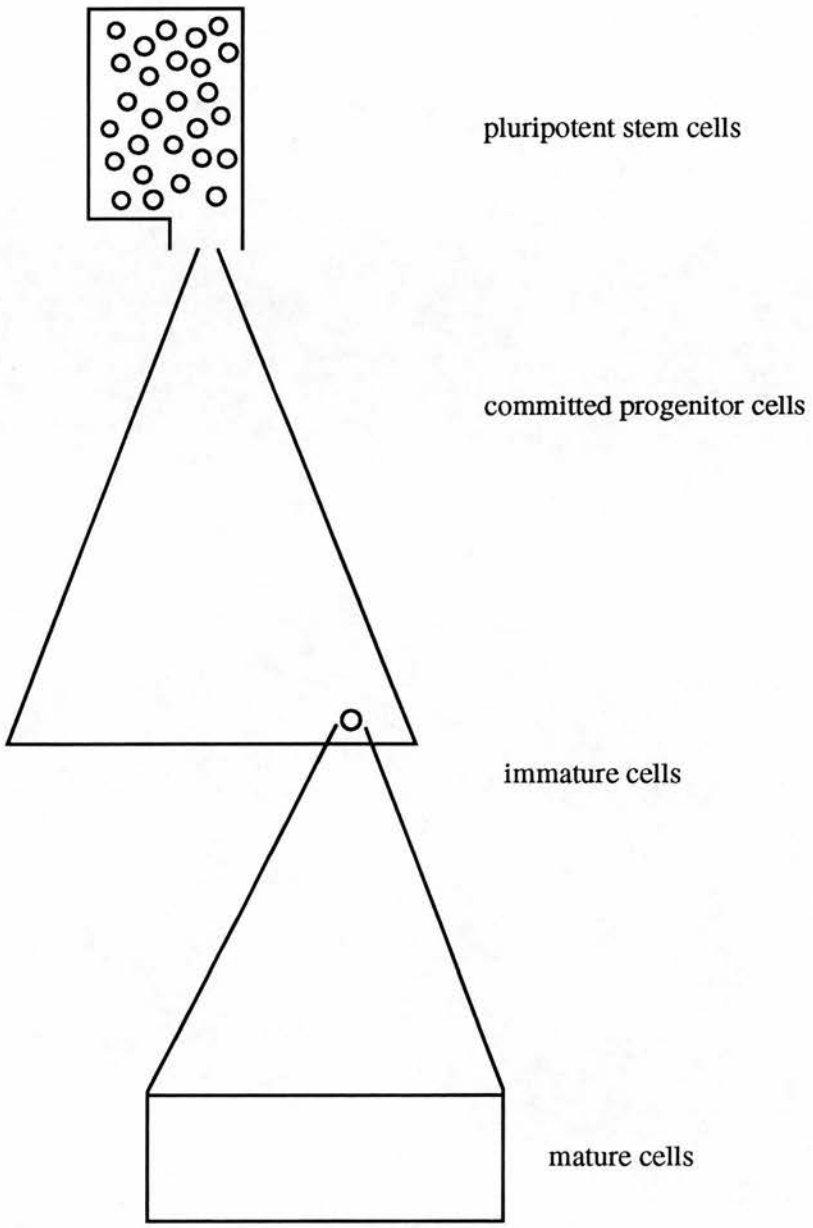

Figure 2: Two dimensional model of the haemopoietic system illustrating how the transient amplifying populations of committed progenitor cells and immature cells can generate large numbers of end stage mature cells from a small number of pluripotent stem cells.

derived from single marrow cells using irradiation-induced chromosomal markers and the cells which gave rise to these colonies became known as colony forming units-spleen (CFU-S). The CFU-S was originally considered to be an assay for murine PHSC but it has since been shown, by the use of cell elutriation to separate PHSC from CFU-S and other progenitors, that long term repopulation of haemopoiesis in irradiated recipients can be obtained without any CFU-S (Jones et al, 1990). In fact CFU-S are a heterogeneous group of multipotential haemopoietic progenitor cells with late day-12 (the time taken to develop colonies) CFU-S representing more primitive progenitors and early day-8 CFU-S representing later more committed progenitors.

However, despite the lack of assays for PHSCs, it was subsequently shown in elegant experiments using chromosomal markers induced either by irradiation (Wu et al, 1968; Abramson et al, 1977) or by retroviral integration (Dick et al, 1985; Keller et al, 1985; Lemischka et al, 1986) that the cells of both the murine myeloid and lymphoid lineages can be derived from a single cell with a high capacity for self-renewal. The ability to reconstitute the lymphohaemopoietic system in irradiated recipients following the infusion of bone marrow cells from either syngeneic or allogeneic donors is now a well-established therapeutic procedure in clinical medicine, recently recognised by the award of a Nobel Prize[1] (Thomas et al, 1975; Santos 1987). Other indirect evidence supporting the existence of PHSCs comes from the demonstration of clonal markers in human blood cell neoplasms (Fialkow, 1980). For example in chronic granulocytic leukaemia (CGL) the marker Philadelphia (Ph) chromosome and glucose-6-phosphate dehydrogenase (G-6-PD) isoenzyme restrictions have been found in cells of the megakaryocyte, erythroid, granulocyte, monocyte and B lymphocyte lineages but not in fibroblasts or other tissues (Richman et al, 1985). Furthermore cases of T lymphoid blast crises have been reported in CGL indicating that the target cell for transformation in this condition probably is a PHSC (Hernandez et al, 1982; Griffin et al, 1983; Chan et al, 1986). In sideroblastic anaemia G-6-PD isoenzyme restriction has been found in haemopoietic cells as well as in both T and

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[1]E Donnall Thomas MD, Nobel Laureate 1990 (Hamblin, 1991).

B lymphoid cells (Prchal et al, 1978) and other disorders where there is evidence to support a clonal defect in pluripotent HSCs include paroxysmal nocturnal haemoglobinuria (Rosse, 1989) and aplastic anaemia (Marsh and Geary, 1991).

2.1.2 Characterisation of Pluripotent haemopoietic stem cells.

The further characterisation of human PHSCs has proved problematic as there is no suitable in vitro assay for PHSCs and because it has been difficult to purify and separate this very low frequency cell population from other cell types. Even extensive experimentation in the murine system, where reconstitution experiments can be more easily undertaken, has only achieved limited enrichment for murine PHSCs (Visser et al, 1984; Spangrude et al, 1991). But despite these limitations considerable information on the nature of putative human PHSCs and how they differ from committed progenitor cells has accumulated from reconstitution data in primate and human bone marrow transplantation (Kaizer et al, 1985; Berenson et al, 1988) and from various in vitro studies (Table 1). These in vitro studies have used combinations of different protocols for cell fractionation to separate mature cells and committed progenitor cells from cell populations containing putative HSCs. These cell fractionation methods have included density separation, counter flow centrifugal elutriation, positive and negative immunoselection, positive selection by flow cytometry and the treatment of cells by S phase specific chemotherapeutic agents such as 4-hydroperoxycyclophosphamide (4-HC), 5-fluorouracil (5-FU) and hydroxyurea (HU). Using these methods, in vitro assays have been described for cells giving rise to blast colonies (CFU-Blast; Leary and Ogawa, 1987; Rowley et al, 1987; Brandt et al, 1988) and for cells giving rise to colonies of high proliferative potential (HPP-CFC; McNiece et al, 1990). These CFCs exhibit properties different to those of committed progenitor cells and may closely resemble PHSCs. Other assay systems have exploited the use of long term bone marrow cultures (LTBMC) describing stroma-dependent blast colony-forming cells (Gordon et al, 1987a) and the initiation of haematopoiesis by the subsequent assay of committed progenitor cells (Winton and Colenda, 1987; Sutherland et al, 1989). Furthermore, novel in vivo assay

systems for human PHSCs have been developed which are able to sustain human haemopoietic engraftment and these include the use of SCID-hu mice (Peault et al, 1991) and the in utero transplantation of sheep with sources of human PHSCs (Srour et al, 1992).

Table 1. Properties discriminating committed progenitor cells from putative pluripotential haemopoietic stem cells[1].

	Cell Type	
Property	Putative PHSC	Committed Progenitor cell
Flow cytometric light scattering characteristics	low forward low orthagonal	higher forward low orthagonal
Surface antigen expression	CD34 + HLA DR - Lin -	CD34 + HLA DR + Lin +
Sensitivity to S-phase chemotherapeutic agents	-	+
Dependence upon CSFs	-	+

[1]for more detailed explanation see text.

These and other studies listed below have indicated that putative human PHSCs a) exhibit low forward and vertical light scatter properties following flow cytometry (Andrews et al, 1989; Verfaillie et al, 1990); b) are positive for the CD34 cell surface antigen but negative for the HLA-DR antigen and other lineage (lin-) specific markers (Keating et al, 1984; Gordon et al, 1985a;) and c) are more resistant to therapy with chemotherapeutic agents such as 4-HC, 5-FU and HU than committed progenitor cells (Siena et al, 1985; Gordon et al, 1985b) indicating that these cells are in G_0 or the resting phase of the cell cycle. Data also

suggest that cells such as the CFU-blast may survive, but not proliferate in the absence of CSFs indicating that PHSCs may not be dependent on CSFs for their survival (Leary and Ogawa, 1987).

In contrast the committed progeny of stem cells (progenitor cells), a) exhibit higher forward light scatter following flow cytometry indicating larger size (Sutherland et al, 1989) (this may reflect exit from G_0); b) are positive for the CD34 and HLA-DR antigens (Civin et al, 1984; Civin et al, 1987; Lu et al, 1987), express activation antigens such as the transferrin receptor, CD71 (Peschel et al, 1989) and CD38 (Terstappen et al, 1991) and begin to express lineage specific markers (Lin+) such as CD33 and CD11b in the case of myeloid progenitors (Andrews et al, 1989; Peschel et al, 1989); c) are sensitive to therapy with agents such as 4-HC and 5-FU and d) require colony stimulating factors and/or interleukins for both their survival and proliferation (Suda et al, 1985). A more detailed description of these cytokines follows on later.

2.1.3. Regulation of PHSCs

The mechanisms whereby PHSCs are regulated to divide and undergo self-renewal or lineage commitment remain poorly understood. However there is evidence, obtained from the study of murine CFU-S, to support the possible negative feedback regulation of PHSCs by a specific stem cell inhibitor (SCI) produced by mature cells. Medium conditioned by either murine or human bone marrow cells has been shown to selectively inhibit the proliferation of primitive murine multipotential haemopoietic cells (CFU-S) but not the proliferation of more developmentally restricted progenitor cells (Lord et al, 1976; Wright et al, 1980). Recently this 'stem cell inhibitor' has been shown to be produced by human macrophages and to be identical to a known cytokine, macrophage inflammatory protein 1-α (MIP-1α), indicating that the mechanisms involved in the regulation of both murine and human HSCs may be identical (Graham et al, 1990). In the case of CFU-S regulation there is evidence to support the existence of a stimulator that switches these cells into the cell cycle (Lord et al, 1977; Toksoz et al, 1980) indicating that the proliferative status for CFU-S or PHSCs may be modulated by the relative levels of an inhibitor and stimulator.

The mechanisms whereby PHSCs undergo either self-renewal or commitment to generate progenitor cells remain unknown. It was originally proposed that the stem cells either underwent self-renewal or became committed to a particular lineage according to a defined probability. This process was believed to occur at random during subsequent cell divisions and to be independent of the previous history of cell divisions. The random nature of this process (labelled "stochastic") was described as being analogous to radioactive decay, whereby following analysis of a large number of events, a regular pattern of haemopoiesis would be achieved (Till et al, 1964). The mechanisms whereby the probabilities of either self-renewal or lineage commitment might be determined were not proposed. Following the observation of a range of multipotent and oligopotent progenitor cells it was then proposed that lineage commitment occurred with a progressive loss of differentiation potentials but still in a stochastic manner (Ogawa et al, 1983).

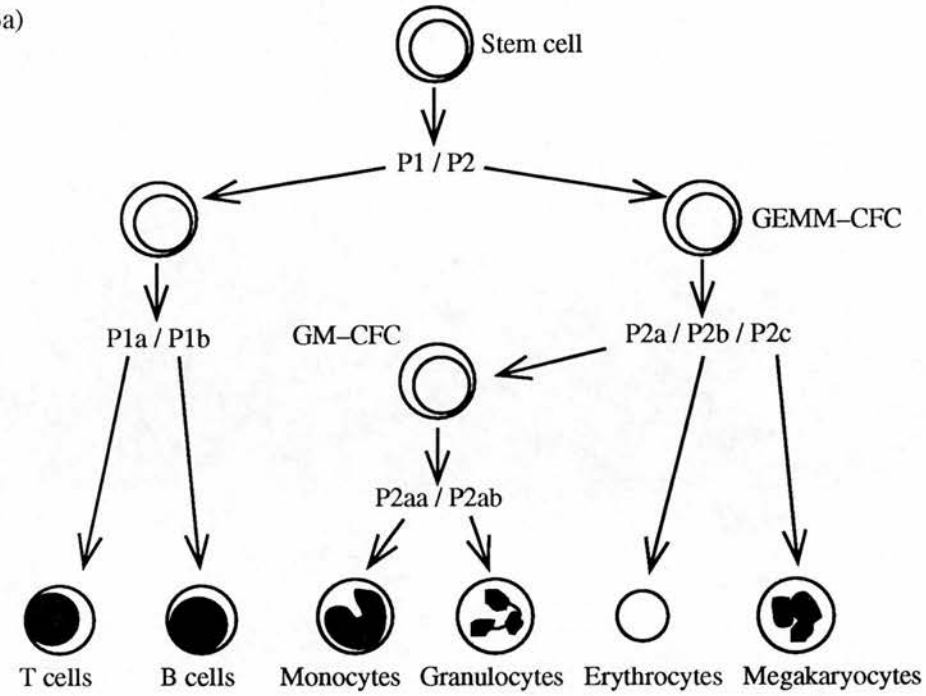
However, other theories have argued that lineage determination in haemopoiesis is not a random process. One viewpoint proposes that environmental influences such as the bone marrow microenvironment (Curry and Trentin, 1966) or the presence of humoral factors (Heyworth et al, 1988) determine the outcome of lineage determination (Figure 3b). Alternatively it has been proposed that lineage determination is genetically pre-determined and occurs in a sequential manner. An early model proposed the sequential losses of differentiation options (Nicola and Johnson, 1982) but a more recent model proposes that lineage potentials in haemopoiesis are expressed individually and in a genetically predetermined sequence as progenitor cells mature (Figure 3c) (Brown et al, 1989). The latter model cites evidence to support the fact that differentiation is genetically programmed within cells and is able to explain how environmental influences interact with expressed lineage potentials to permit differentiation along one pathway of maturation. Another feature of this model, quite distinct from others, is the close developmental relationship between certain lineages such as megakaryocytes and erythrocytes, granulocytes and monocytes and monocytes and B cells (Brown et al, 1990).

Figure 3 Lineage determination in haemopoiesis.

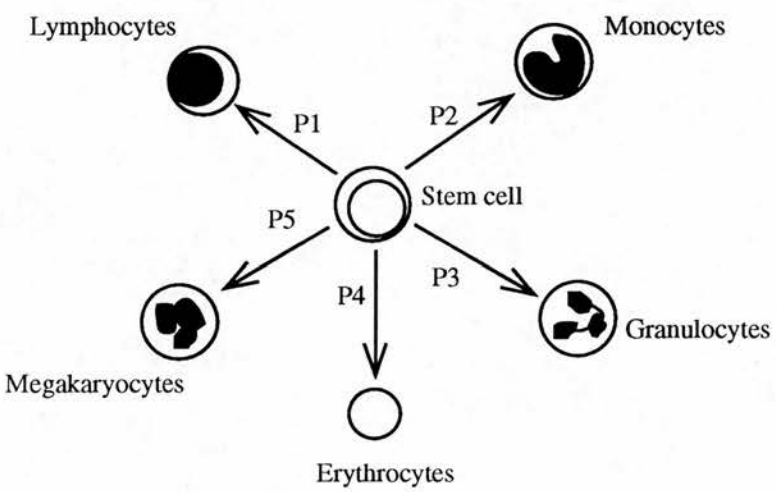
Figure 3a illustrates a conventional representation of decision points during the diversification of haemopoietic progenitor cells. Differentiation pathway options are designated p1, p2, etc. GEMM-CFC and GM-CFC are colony forming cells restricted to granulocyte/erythrocyte/macrophage/megakaryocyte differentiation and granulocyte/macrophage differentiation respectively. Figure 3b illustrates a multipotent haemopoietic stem cell where differentiation options p1-p5 (possibly induced by environmental influences such as the local presence of haemopoietic growth factors) are simultaneously and equally available. Figure 3c illustrates an ordered (genetically predetermined) process of progenitor cell development. Differentiation options p1-p6 are expressed individually and in a preferred order. Cells gradually acquire one differentiation potential followed by loss of this capacity as the next differentiation option is expressed (Adapted from Brown et al, 1990).

The direct evidence to support these theories of lineage commitment in haemopoiesis will require experiments to be performed on single PHSCs to enumerate the number of lineage options available within a single stem cell at a given time point.

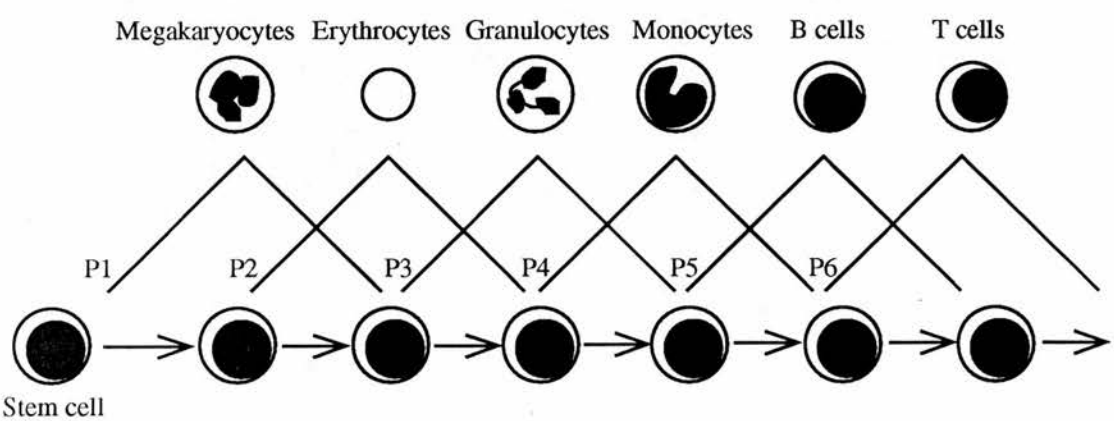
Figure 3a)


Stem cell
P1 / P2
GEMM–CFC
P1a / P1b
GM–CFC
P2a / P2b / P2c
P2aa / P2ab
T cells
B cells
Monocytes
Granulocytes
Erythrocytes
Megakaryocytes

b)


Lymphocytes
Monocytes
P1
P2
Stem cell
P5
P4
P3
Granulocytes
Megakaryocytes
Erythrocytes

c)


Megakaryocytes
Erythrocytes
Granulocytes
Monocytes
B cells
T cells
P1
P2
P3
P4
P5
P6
Stem cell

2.2 Haemopoietic Progenitor Cells

Haemopoietic progenitor cells were first recognised when colonies of mature granulocytes and macrophages were grown from individual haemopoietic cells which had been placed in semi-solid medium containing appropriate colony-stimulating activity (Bradley and Metcalf, 1966; Pluznik and Sachs, 1966). This demonstration of in vitro factor dependent colony growth of haemopoietic progenitor cells (or colony forming cells (CFCs)) stimulated intense research activity which resulted in the discovery of haemopoietic progenitor cells for other lineages and the identification of the soluble factors which stimulated the growth of colonies from these colony forming cells. The use of karyotypic markers, G-6-PD isoenzyme studies and the growth of colonies from single manipulated cells confirmed the clonal nature of haemopoietic colonies (Singer et al, 1979; Metcalf et al, 1980) and the progenitor basis of human haemopoiesis is now well established (Figure 4); Nathan, 1990; Pike and Robinson, 1970; Tepperman et al, 1974; Iscove et al, 1974; Clarke and Housman, 1977; Fauser and Messner 1979; Ash et al, 1981; Messner et al, 1982.

Haemopoietic progenitor cells, which are the descendants of PHSCs, act as transit amplifying populations by exhibiting properties of a limited self-renewal capacity, commitment to a restricted range of lineages and both proliferation and differentiation in response to appropriate CSFs. Progenitor cells eventually become restricted to a single lineage and ultimately generate mature cells which are terminally differentiated and which have no further potential for self-renewal. The ability of progenitor cells to proliferate and differentiate in response to a range of lineage restricted or specific CSFs offers a mechanism whereby haemopoietic lineages can be amplified in response to the increased CSF concentrations which occur in pathological conditions such as tissue hypoxia, infection or chemo-radiation induced cytopenia (Cannistra and Griffin, 1988). Furthermore, the recent cloning of CSFs and other cytokines involved in the regulation of haemopoiesis has permitted the opportunity to use these agents clinically in the treatment of patients with disordered haemopoiesis (Moore et al, 1991a).

Figure 4

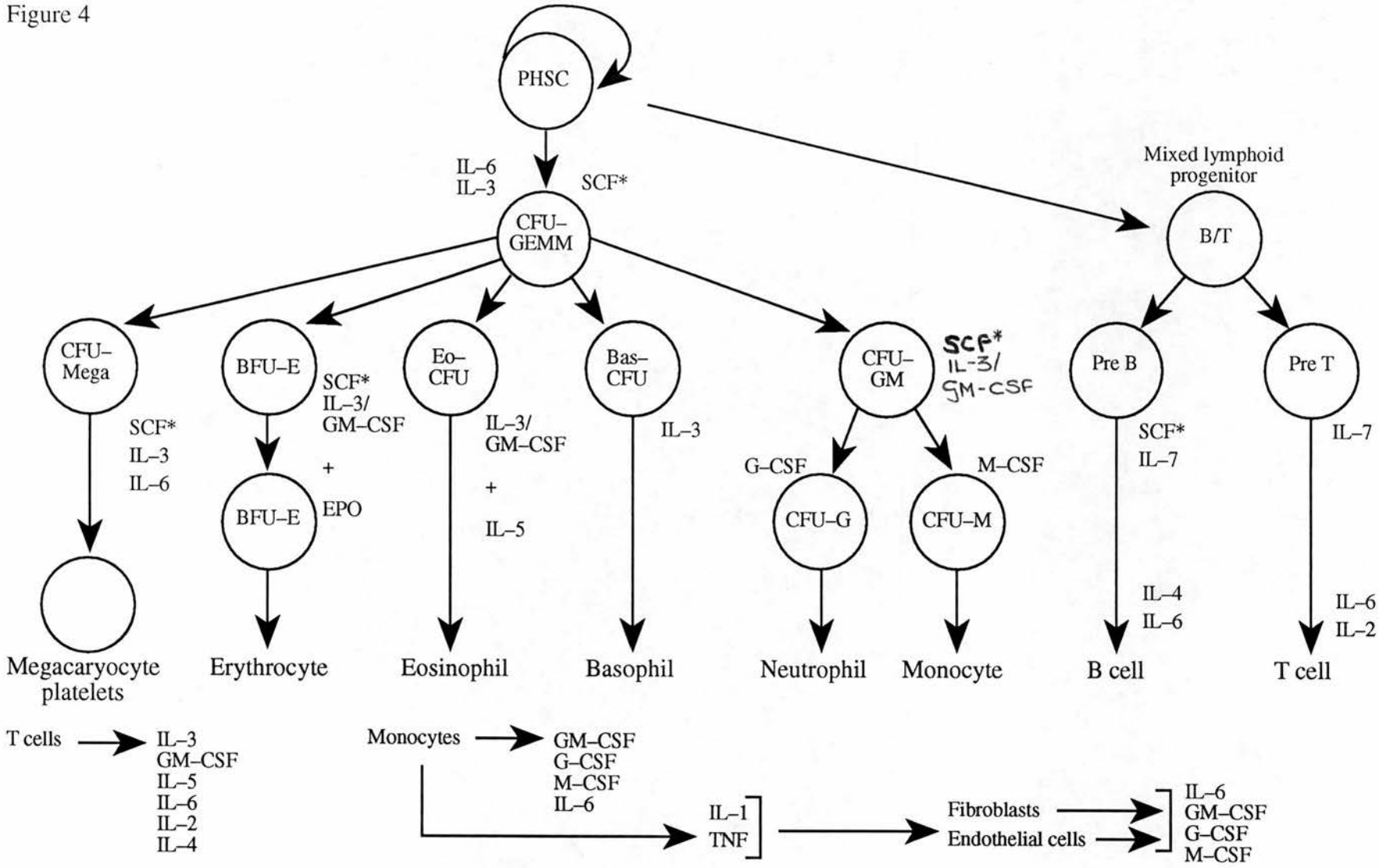

PHSC
IL–6
IL–3
SCF*
CFU–
GEMM
Mixed lymphoid
progenitor
B/T
CFU–
Mega
SCF*
IL–3
IL–6
BFU–E
SCF*
IL–3/
GM–CSF
+
EPO
BFU–E
Eo–
CFU
IL–3/
GM–CSF
+
IL–5
Bas–
CFU
IL–3
CFU–
GM
SCF*
IL-3/
GM-CSF
G–CSF
M–CSF
CFU–G
CFU–M
Pre B
Pre T
SCF*
IL–7
IL–7
IL–4
IL–6
IL–6
IL–2
Megacaryocyte
platelets
Erythrocyte
Eosinophil
Basophil
Neutrophil
Monocyte
B cell
T cell
T cells
IL–3
GM–CSF
IL–5
IL–6
IL–2
IL–4
Monocytes
GM–CSF
G–CSF
M–CSF
IL–6
IL–1
TNF
Fibroblasts
Endothelial cells
IL–6
GM–CSF
G–CSF
M–CSF

Figure 4. The progenitor basis of haemopoiesis.

The figure demonstrates a differentiation map of myeloid progenitor cells beginning with the pluripotent haemopoietic stem cell. Although the existence of these progenitor cells is well established, their exact positions in the differentiation map in relation to each other and to the PHSC remains unresolved. In contrast, the lymphoid progenitors remain poorly defined but they have been included for completeness. CFU-GEMM = colony forming unit - granulocyte, erythrocyte, megakaryocyte, monocyte; CFU-Meg = colony forming unit-megakaryocyte; BFU-E = blast forming unit - erythroid; CFU-E = CFU-erythroid; CFU-Eo = CFU-eosinophil; CFU-Bas = CFU-basophil; CFU-GM = CFU-granulocyte, monocyte; CFU-G = CFU-granulocyte; CFU-M = CFU-monocyte. The factors that stimulate the growth and differentiation of particular progenitors are shown to the right of the lines connecting the progenitors to their more mature progeny. The asterisks next to stem cell factor (SCF) indicate provisional stimulatory activities based on early experimental data. There appears to be a hierarchy of development with SCF, IL-6 and IL-3 acting on early multipotent myeloid progenitors, GM-CSF acting to a weaker extent on early multi-lineage progenitors but more strongly on a broad range of later progenitors and Epo, G-CSF, M-CSF and IL-5 acting on later progenitors. The effects of IL-3 and GM-CSF on lineage specific progenitors are seen primarily in synergy with late acting factors such as Epo, G-CSF, M-CSF and IL-5: (For further explanation see Figure 5 and text). The principal tissue sources of these positive regulatory cytokines are shown at the bottom of the figure. Negative regulators of haemopoiesis are not shown because their actions are complex and their physiological significance remains to be determined.

2.3 Cytokines involved in haemopoietic progenitor cell regulation.

The factors or cytokines that regulate haemopoiesis were originally identified as constituents of supernatants derived from heterogeneous cell populations or cell lines and their effects were detected in bio-assays on target populations. Many of these factors have now been purified and their genes cloned and it has become evident that they have many actions, including the induction of secondary cytokines, which overlap with the actions of other cytokines. Individual cytokines had previously been allocated more than one name when they had displayed different actions on different target cell populations but an international nomenclature has now been adopted. The adopted system includes the colony-stimulating factors (CSFs), the interleukins and a group of miscellaneous cytokines, many of which are negative regulators. These are described in more detail below. The CSFs promote the growth, differentiation and survival of haemopoietic progenitor cells, particularly those in the myeloid lineages and in addition enhance a variety of functional properties of mature haemopoietic cells. The interleukins are another group of cytokines that were originally believed to be produced by lymphocytes and macrophages and which were thought to act primarily on B or T lymphoid cells. However it is now known that interleukins are produced by a broad spectrum of cell types and that these cytokines can in many circumstances, act on cells other than those in the lymphoid lineage. The cytokines that stimulate the growth and differentiation of particular haemopoietic progenitors are shown in Figure 4 and Tables 2 and 3 list key biochemical and physical properties of well-characterised human cytokines.

2.3.1. Interleukin 3

Interleukin 3 (IL-3) is produced from activated T cells and was originally known as multi-CSF on account of its ability to support the survival, self-renewal, proliferation and differentiation of a wide range of early haemopoietic progenitors including the mixed myeloid progenitor CFU-GEMM, the megakaryocyte-CFC, the granulocyte macrophage- CFC and the early erythroid progenitor, BFU-E. In the presence of IL-3 these progenitors are able to develop into many different cell types such as

Table 2. **Biochemical and physiological properties of principal myeloid haemopoietic growth factors[1].**

Name	Main cellular source	Chromosomal location	MW(Kd)	Principal Actions
IL-3	T cells	5q23-31	14 - 28	multi-lineage myeloid growth and maturation factor.
GM-CSF	T cells endothelial cells fibroblasts macrophages.	5q23-31	14 - 35	neutrophil/eosinophil/monocyte growth maturation factor.
G-CSF	endothelial cells fibroblasts macrophages.	7q11.2-21	18 - 22	neutrophil growth and maturation factor.
M-CSF	endothelial cells	1p13-21[2]	70 - 90 (homo dimer)	monocyte growth and maturation factor
IL-5	T cells	5q31	32 - 62	eosinophil growth and maturation factor
Epo	kidney	7q11-22	34 - 39	erythrocyte growth and maturation factor
IL-6	T cells endothelial cells fibroblasts	7p21	21 - 28	multifunctional cytokine mediator of host response to injury. megakaryocyte growth and maturation factor.
SCF	Bone marrow stroma	unknown	18	multilineage growth factor

[1] for more detailed explanation see text. [2] previously assigned to 5q33.1; for updated location see ref.(Morris et al, 1991)

Table 3. **Biochemical and physiological properties of other well characterised human interleukins[1].**

Name	Main cellular source	Chromosomal locations	MW(Kd)	Principal actions
IL-1	macrophages endothelial cells fibroblasts	2q13-21	17	multifunctional cytokine trigger of host response to injury
IL-2	T cells	4q26-27	15	stimulator of T cell mediated immune response
IL-4	T cells	5q23-32	20	B cell growth and differentiation factor
IL-7	marrow, thymic, splenic stroma	unknown	20 - 28	B, T cell growth factor
IL-9	T cells	5	20 - 30	erythroid growth factor
IL-11	marrow stroma	unknown	23	megakaryocyte maturation factor

[1] for more detailed explanation see text

early erythrocytes, neutrophils, eosinophils, basophils, macrophages and megakaryocytes (Clark and Kamen, 1987). IL-3 has been shown to support the proliferation of the earliest identifiable multi-lineage progenitors in both mouse and man and to display synergy with IL-6 in this property (Leary et al, 1988). Furthermore IL-3 has been shown to exhibit synergism with G-CSF and possibly with GM-CSF to enhance the proliferation of committed myeloid progenitors (Paquette et al, 1987, Sieff et al, 1987). Both mature neutrophils and macrophages and their precursors express IL-3 receptors and enhanced survival and function of mature cells in the presence of IL-3 has been documented in vitro (Whetton and Dexter, 1989). The observations of the effects of GM-CSF, G-CSF, M-CSF, IL-5 and erythropoietin (vide infra) suggest a hierarchical role for the CSFs with IL-3 acting predominantly on early multi-lineage progenitors, GM-CSF acting to a weaker extent on early multi-lineage progenitors and more strongly on a broad range of later progenitors and G-CSF, M-CSF, IL-5 and erythropoietin (Epo) acting later still to support the growth of late progenitors that are already committed to their particular lineages (Figure 4, Figure 5).

The gene for IL-3 is located on the long arm of chromosome 5 along with the genes coding for GM-CSF, IL-5, IL-4 and the M-CSF receptor. Early studies on the in vivo administration of recombinant human (rh) IL-3 in humans have shown great promise by producing a multi-lineage response with an increase in leucocytes (eosinophils, neutrophils and lymphocytes), platelets and reticulocytes. The subsequent administration of GM-CSF following IL-3 has produced a further synergistic leucocyte response (Hoelzer et al, 1991). IL-3 probably plays an important role in the maintenance of steady state haemopoiesis but a more limited role in the haemopoietic response to stress in contrast to the more lineage restricted CSFs - GM-CSF, G-CSF, M-CSF and Epo (Figure 6; Cannistra and Griffin, 1988).

2.3.2. Granulocyte-monocyte colony-stimulating factor

In vitro, granulocyte-monocyte colony stimulating factor (GM-CSF) primarily supports the proliferation and differentiation of macrophage, neutrophil and eosinophil colonies and colonies containing both

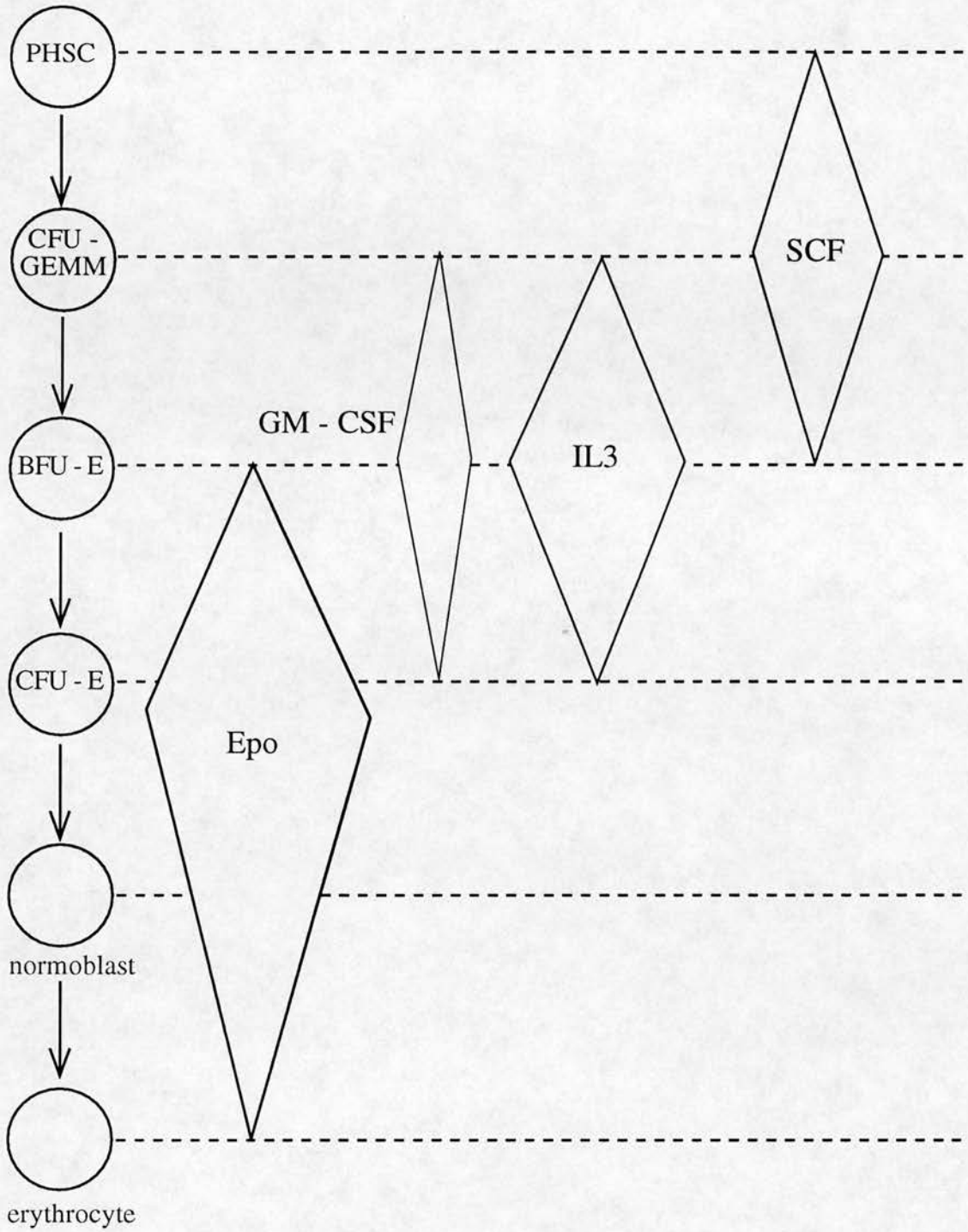


Figure 5: Illustration of the developmental hierarchy of responsiveness of haemopoietic progenitor cells to different growth factors. The erythroid lineage is shown as an example. The ability of factors to exert their maximal effects at different stages of maturation permits a synergistic response with factor combinations. For example the in vitro combination of IL–3 and Epo is able to generate much larger numbers of normoblasts and erythrocytes from a single BFU–E progenitor than when either factor is used alone.

neutrophils and macrophages. These effects are mediated by direct actions on the colony forming cells (CFC) - GM-CFC, M-CFC, G-CFC and Eo-CFC (Metcalf, 1986). In addition GM-CSF can also in vitro promote the formation of a) mixed myeloid colonies indicating CSF activity towards the mixed myeloid progenitor, CFU-GEMM (Sieff et al, 1985) and b) erythroid colonies in the presence of erythropoietin indicating activity towards the early erythroid progenitor, BFU-E (Sieff et al, 1985, Donahue et al, 1985). However the CSF activity directed towards mixed myeloid and early erythroid progenitors is less than that observed for IL-3 (Sieff et al, 1987). Studies of the addition of GM-CSF to cultures grown in the presence of IL-3 have produced conflicting results. One study showed no increase in the number of either BFU-E or CFU-GM colonies produced suggesting that most if not all GM-CSF responsive progenitors also respond to IL-3 (Sieff et al, 1987). However another study demonstrated enhanced G-M colony formation when both factors were present suggesting synergy between IL-3 and GM-CSF for the growth of committed myeloid progenitors (Paquette et al, 1987). The in vivo response to rhGM-CSF supports the factor's net dominant actions on the later progenitors by producing dose dependent increases in neutrophils, monocytes and eosinophils but not in reticulocytes or platelets (Moore, 1991a). However the sequential in vivo administration of rhGM-CSF has resulted in marked synergistic leucocyte responses that are encouraging (Hoelzer et al, 1991). The administration of rh GM-CSF in vivo, in contrast to rh G-CSF, generates significant dose dependent side effects and these may be related to GM-CSF's known capacity to generate the secondary production of cytokines and other inflammatory mediators such as IL-1, TNF and prostaglandins from activated monocytes.

CSFs also typically enhance the functional activity of mature cells and the actions of GM-CSF on mature eosinophils, neutrophils and monocytes are well described (Moore, 1991a). GM-CSF is produced by endothelial cells, fibroblasts, and also monocytes and T cells largely in response to inflammatory mediators such as IL-1 and TNF. The combination of its local effect on mature cells and systemic effect on myelopoiesis offers a mechanism whereby an appropriate response to stress can be mounted (Figure 6; Cannistra and Griffin, 1988).

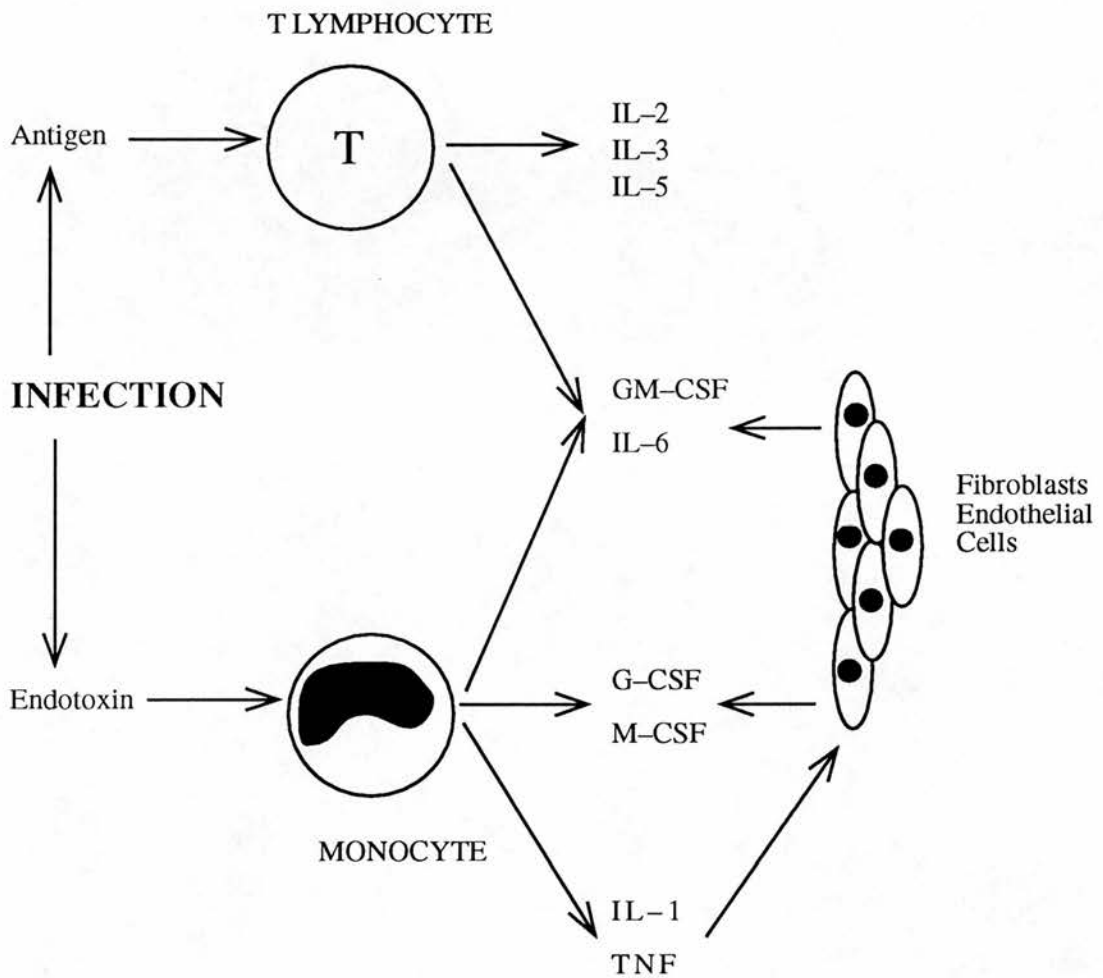


Figure 6: Simple schematic illustration of the haemopoietic cytokine response to infection. The combination of the local and systemic production of the myeloid haemopoietic cytokines IL–6, IL–3, IL–5, GM–CSF, G–CSF and M–CSF not only permits the increased production of granulocytes and monocytes from bone marrow but also enhances the maturation, survival and functional properties of mature cells. IL–2 release activates T and B cell mediated immune responses.

2.3.3. Granulocyte colony-stimulating factor

Granulocyte colony-stimulating factor (G-CSF) is a lineage specific CSF which promotes the growth and differentiation of committed neutrophilic granulocyte progenitors (CFU-G) and which also enhances the survival and function of mature neutrophils. The gene for G-CSF is located on chromosome 7 (7q 11.2-2.1) and G-CSF is produced from endothelial cells and fibroblasts in response to IL-1 and TNF production from monocytes activated following an inflammatory stimulus. G-CSF can also be produced from activated monocytes themselves (Moore, 1991a). The in vivo administration of recombinant human (rh) G-CSF results in neutrophil leucocytosis and has been highly successful in the treatment of chemotherapy induced neutropenia, congenital, chronic idiopathic and cyclical neutropenia syndromes (Moore, 1991a) and also drug induced agranulocytosis (Patton et al, 1992). G-CSF influences the functions of mature neutrophils by enhancing their ability to phagocytose particles, to produce superoxide anions in response to the bacterial peptide f-Met-Leu-Phe and to kill tumour targets through antibody-dependent cellular cytotoxicity (Clark and Kamen, 1987). The observation in the murine system that G-CSF can act on early progenitors by synergising with IL-3 to increase megakaryocyte colony formation has not been demonstrated in humans (McNiece et al, 1988) although G-CSF has been shown to synergise with IL-3 in the production of total colonies produced from committed myeloid progenitors (Sieff et al, 1987: Paquette et al, 1987).

2.3.4. Macrophage colony-stimulating factor

Macrophage colony-stimulating factor (M-CSF) is a lineage specific CSF which promotes the growth and differentiation of committed macrophage progenitors (CFU-M) but this proliferative property of human M-CSF is much weaker than that observed for human M-CSF acting on murine macrophage progenitors. This indicates that the main function of human M-CSF may be as a survival and activating factor for mature monocytes and macrophages rather than as a proliferation and differentiation factor for their precursors (Clark and Kamen, 1987). In response to M-CSF macrophages have demonstrated enhanced survival,

increased phagocytosis and intracellular killing of micro-organisms, enhanced tumour cell killing and the production of cytokines such as GM-CSF, prostaglandin-E and α-interferon (Whetton and Dexter, 1989). M-CSF is produced from endothelial cells and fibroblasts in response to IL-1 and TNF production from monocytes activated following an inflammatory stimulus. However M-CSF can also be produced from monocytes themselves in response to inducers such as GM-CSF and α-INF indicating that an autocrine mechanism may act to control monocyte/macrophage function during the inflammatory response. M-CSF binds to a high affinity cell surface receptor that is expressed on committed precursor cells but to a much higher degree on macrophages. The M-CSF receptor (M-CSFR) is encoded by the c-fms proto-oncogene which is located at 5q33.3, and is a transmembrane glycoprotein with an intracellular tyrosine kinase domain.

Recombinant human M-CSF (rhM-CSF) is currently being administered in a phase 1 study to patients with metastatic melanoma. Dose dependent increases in circulating monocytes, which are morphologically large and extensively vacuolated, have been observed along with modest decreases in platelet counts. Enhanced functional activity of monocytes has also been demonstrated along with clinical anti tumour effects and the treatments have been tolerated well. Other potential indications advocated for rhM-CSF therapy include therapy for invasive fungal infections and differentiation therapy for acute myeloid leukaemias and myelodysplastic syndromes. Recombinant human M-CSF has also been shown to shorten the period of chemotherapy induced neutropenia but this effect is probably mediated by the release of secondary cytokines from activated monocytes such as G-CSF and GM-CSF (Bajorin et al, 1991).

2.3.5. Interleukin 5

Interleukin 5 (IL-5) is produced from T cells and is a lineage specific CSF for committed eosinophil progenitors which also enhances the functional activity of mature cells. The human gene for IL-5 is located at 5q31. Both IL-3 and GM-CSF can also stimulate eosinophil colonies in vitro as they are multi-lineage in their effects but the regulation of eosinophilia in

pathological circumstances remains poorly understood. In addition to its effects on eosinophils, murine IL-5 is also a B cell growth factor but these properties have not been demonstrated for human IL-5 (Sanderson et al, 1988).

2.3.6. Erythropoietin

Erythropoietin (Epo) is a major growth factor responsible for the regulation of erythropoiesis. It is a true hormone whose existence has been postulated for many years (Carnot and Deflandre, 1906). It is secreted into the blood from proximal peritubular cells in the kidney in response to anaemia and/or tissue hypoxia and acts on erythroid precursors in the marrow via a recently identified high affinity receptor (Hambley and Mufti, 1990; D'Andrea and Jones, 1991). Its main site of action is on the late erythroid progenitor, CFU-E, which is only responsive to Epo and not to other growth factors. Epo enhances the proliferation, differentiation and maturation of CFU-E and its progeny to generate small colonies of erythrocytes (≈ 120 cells) and offers the mechanism whereby increased demands for erythropoiesis can be met. The early erythroid progenitor, BFU-E, is stimulated by IL-3 or GM-CSF and to a much lesser extent by high concentrations of Epo to divide and generate CFU-E that can then become responsive to Epo. Therefore Epo in combination with IL-3 or GM-CSF ultimately generates very large colonies of differentiated erythroblasts (≥2000 cells) which are in fact a collection of multiple smaller CFU-E colonies (Nathan, 1990; Sieff et al, 1987). The human gene for Epo resides on chromosome 7 and recombinant human (rh) Epo was the first haemopoietic growth factor licensed for clinical use. It has proved strikingly effective for the treatment of anaemia associated with end stage renal failure and is currently being evaluated in other areas such as the anaemia of prematurity, autologous blood transfusion, the anaemia of chronic disease, human immunodeficiency virus associated anaemia and anaemias associated with primary haematological disorders such as myeloma and the myelodysplastic syndromes (MDS) (Erslev, 1991; Davis & Morstyn, 1992). The demonstration in the mouse of megakaryocyte maturational properties due to Epo has not been shown in the human haemopoietic system (Ishibashi et al, 1987).

2.3.7. Stem Cell Factor (SCF) or c-kit ligand

Stem cell factor is a recently identified haemopoietic growth factor of great importance whose role in human haemopoiesis remains to be fully defined. SCF is a stromal derived factor required for murine PHSCs that is the product of the Steel (Sl) locus and the ligand for the tyrosine-kinase receptor c-kit that is the product of the murine W locus. Mice with homozygous defects at either the W or Sl loci exhibit defective haemopoiesis, granulopoiesis and melanogenesis indicating that the c-kit ligand/receptor interaction is of fundamental importance to the development of these organ systems (Zsebo et al, 1990; Bernstein et al, 1991). The bone marrow of mutant Sl mice can reconstitute the lymphohaemopoietic system of unirradiated W mutant mice, and SCF has been shown to exhibit potent multi-lineage activity on early murine myeloid and lymphoid progenitors (McNiece et al, 1991a; Williams et al, 1992). In the human system recombinant human SCF does not promote haemopoietic colony formation when used alone, but in combination with other CSFs such as IL-3 or GM-CSF or Epo, SCF stimulates the growth of greater numbers of colonies that are of increased size than those seen when the CSFs are used alone (McNiece et al, 1991b). This synergistic activity of SCF with other CSFs is present on CD34+ lineage negative cells - the earliest identifiable haemopoietic precursors (Bernstein ID et al, 1991; Brandt et al, 1992). However whether this activity is present on cells within this group which are true PHSCs remains to be determined. Early in vivo studies in primates have shown that rhSCF stimulates multi-lineage haematopoiesis by increasing the numbers of peripheral blood erythrocytes, lymphocytes, monocytes, neutrophils, eosinophils and basophils. Increases in marrow megakaryocytes were consistently observed but the observed increases in platelet counts were inconsistent (Andrews et al, 1991). These preliminary data indicate that SCF is a powerful proliferative stimulus for multipotent haemopoietic progenitor cells and possibly for PHSCs. Its interactions with other haemopoietic cytokine regulators and its ultimate role in human haemopoiesis remain to be defined but SCF would appear to be an agent with great therapeutic potential.

2.3.8. Interleukin 6

Interleukin 6 (IL-6) is a recently characterised multi-functional cytokine that plays a pivotal role in the co-ordinated host response to injury (Van Snick, 1990). Both IL-1 and TNF, the initial triggers of the host response to injury are potent inducers of IL-6 that can be released from many cell types including fibroblasts, endothelial cells, macrophages and keratinocytes. Other IL-6 inducers include primary factors such as viral infection and bacterial lipopolysaccharide. IL-1 and IL-6 share some properties and synergy, for example in the production of IL-2 from activated T cells, and it is probable that many of the functions originally attributed to IL-1 are due to IL-6 (Ikebuchi et al, 1988). Following IL-6 release, acute phase protein synthesis is initiated in the liver, ACTH is released from the pituitary gland, T cells and haemopoietic precursors become responsive to their growth and differentiation factors and antibody production is stimulated in activated B cells. Other properties include keratinocyte, mesangial cell and B cell proliferation and the abnormal production of IL-6 is implicated in the pathogenesis of many diseases including plasma cell neoplasms, mesangial proliferative glomerulonephritis, psoriasis, rheumatoid arthritis, and AIDS.

The IL-6 gene is located on chromosome 7 and both cis and trans regulating factors are involved in the regulation of IL-6 gene expression (Hirano et al, 1990). The IL-6 receptor (IL-6R) is an 80 Kd glycoprotein that is widely expressed on many cell types in accordance with its multiple functional properties. It possesses structural similarities to the IL-1R with an extra cellular region bearing homology with a domain of the immunoglobulin superfamily and an intracellular portion that does not possess a tyrosine kinase domain. Signal transduction is believed to be mediated by an associated non covalently linked 130Kd glycoprotein (Hirano et al, 1990; D'Andrea and Jones, 1991).

However of special interest are the effects of IL-6 on haemopoietic cells. IL-6 has been shown to act in synergy with IL-3 in promoting the proliferation of human multipotential blast cell colonies (Ikebuchi et al, 1987; Leary et al, 1988) and this may be due to IL-6 recruitment of primitive progenitors into cell cycle. IL-6 also has potent thrombopoietic

maturational properties in mice in vitro (Ishibashi et al, 1989a; Quesenberry et al, 1991) and IL-6 induces an increase in platelet number in vivo in both mice and monkeys (Ishibashi et al, 1989b; Asano et al, 1990). The importance of IL-6 in human megakaryopoiesis has recently been confirmed by the demonstration of in vitro synergy with IL-3 in the stimulation of megakaryocyte colonies (Imai et al, 1991) and by the finding of elevated serum IL-6 levels in patients with reactive thrombocytosis (Hollen et al, 1991). The clinical application of IL-6 or inducers of IL-6 such as IL-1 possibly in combination with IL-3 or GM-CSF for the treatment of hypoproliferative thrombocytopenia can be expected in the near future.

2.3.9. Interleukin 1

Interleukin 1 (IL-1) release along with TNF is an essential trigger for the subsequent co-ordinated host response to injury (Bagby, 1989; Starnes, 1991). IL-1 is principally released by activated macrophages in response to invasive stimuli but it can be produced by many cell types including endothelial cells, fibroblasts, epidermal cells and smooth muscle cells. Factors such as cell to cell contact, possibly mediated by cell adhesion molecules, and other cytokines can also induce IL-1 production (di Giovine and Duff, 1990). IL-1 induction from stimulated cells can be significantly inhibited by pre-treatment of cells with glucocorticoids and IL-4.

IL-1 is able to affect haemopoiesis and orchestrate the host response to injury via the production of secondary cytokines. It acts on fibroblasts and endothelial cells to induce IL-6, GM-CSF, IL-3, G-CSF, M-CSF, PDGF and also the autocrine production of further IL-1. IL-1 has been claimed to act in synergy with IL-3 on multi-lineage haemopoietic progenitor cells but it has proved difficult to confirm whether these effects are due to the direct actions of IL-1 on progenitor cells or to the indirect actions mediated by the release of secondary cytokines (Leary et al, 1988; Ikebuchi et al, 1988; Kobayashi et al, 1991). IL-1 also acts on endothelial cells to produce plasminogen activator inhibitor and von Willebrand factor and is a potent stimulator of bone resorption (Haworth, 1989).

IL-1 activity is due to two molecules IL-1α and IL-1ß that have identical properties and act through a common 70Kd high affinity receptor that is expressed on most somatic cells. IL-1α and IL-1ß share 26% amino acid homology and are the products of separate genes located on chromosome 2. It has been proposed that IL-ß evolved by reverse transcription of IL-1α during evolution. Of interest is the observation that IL-1 protects mice from the irradiation-induced lethal haemopoietic syndrome when the IL-1α is given before irradiation but the mechanism of this radioprotective effect remains poorly understood (Neta, 1990).

2.3.10. Interleukin 2.

Interleukin 2 (IL-2), originally described as T cell growth factor, is the main stimulator of the T cell mediated immune response. IL-2 is produced by helper T cells that become activated in response to antigen binding in association with antigen presenting cells (APC) (Pierce, 1989). The activation process, although dependent on IL-1 and IL-6 production from APC, is extremely complex and is the result of poorly understood multiple surface receptor/ligand interactions. Following antigen activation, helper T cells produce IL-2, start to express its receptor and are induced to proliferate. Further actions include the production of other cytokines such as interferons and IL-5, increased activity of non-MHC restricted cytotoxic effector cells such as natural killer (NK) cells and lymphokine-activated killer (LAK) cells and the proliferation and differentiation of antigen-stimulated B cells. The IL-2 receptor is now well characterised. The high affinity receptor is composed of two non covalently linked peptide subunits (p55Kd α and p70Kd ß) which can independently bind IL-2 with lower affinity (Hamblin, 1988). A single copy of the human IL-2 gene exists on chromosome 4 and the gene has been successfully cloned. Recombinant human IL-2 has been widely used in vitro in the study of the T cell immune response and in vivo as immunotherapy for cancer patients (Rees, 1990).

2.3.11. Interleukin 4

Interleukin 4 (IL-4) is a product of activated T cells that was originally described as a potent cofactor for the stimulation and differentation of B

cells. Particular properties included the stimulation of IgG and IgE isotype switching within activated B cells. IL-4 has been subsequently shown to co-stimulate T cell proliferation in conjunction with phorbol esters, to enhance the antigen presenting ability of some macrophages and to synergise with IL-3 in the production of murine mast cells. These properties of IL-4 of the stimulation of IgE and mast cell production have suggested a crucial role for IL-4 in the regulation of the allergic response (Paul, 1991). Other murine studies have indicated that IL-4 is synergistic with lineage specific CSF, for the in vitro proliferation of myeloid, erythroid, megakaryocyte and multi-lineage progenitors (Peschel et al, 1987) but these properties may not be of physiological significance as the observed effects were sub optimal to those of IL-3. The role of IL-4 in human haemopoiesis remains unknown.

2.3.12. Interleukin 7.

Interleukin 7 (IL-7) was first described in the mouse as a stromal cell derived factor, called lymphopoietin-1, which was required for the growth of pre-B cells. Both the murine and human genes have been cloned and IL-7 has been shown to stimulate the growth but not differentiation of both pre-B cells and T cell precursors. Myeloid precursor cells and mature B cells are unresponsive to IL-7 but mature T cells will respond to IL-7 in the presence of a secondary stimulus such as antigen or mitogen (Pierce, 1989; Henney, 1989; Namen et al, 1990). Additional early data from murine reconstitution experiments also suggests a possible role for IL-7 in platelet production (Namen et al, 1990).

2.3.14 Interleukin 9

Interleukin 9 (IL-9) is a cytokine originally purified from a murine T-cell line and the cDNA encoding the human homologue has been isolated from a human leukaemic cell line (Yang et al, 1989; Mock et al, 1990; Renauld et al, 1990). IL-9 has been mapped to human chromosome 5 and early in vitro studies have shown that IL-9, in combination with Epo, is able to support the growth of early IL-3 responsive BFU-E (Donahue et al, 1990). Further characterisation of this cytokine is awaited.

2.3.14 Interleukin 11.

Interleukin 11 (IL-11) is a cytokine recently cloned from an immortalised primate bone marrow stromal cell line, the cDNA of which shows 97% identity with a cDNA sequence isolated from a human fetal fibroblast cell line (Paul et al, 1990). Recombinant human IL-11, in combination with IL-3, enhances human megakaryocytopoiesis in vitro (Bruno et al, 1991; Teramura et al, 1992) and rhIL-11, when used alone, is able to increase megakaryocyte ploidy, indicating that IL-11 has thrombopoietin activity. In vivo, in rats, rhIL-11 shows a dose dependent increase in platelet numbers which appears to be mediated by the promotion of megakaryocyte maturation (Yonemura et al, 1993). Further characterisation of this potentially important cytokine is awaited.

2.3.15. Leukaemia inhibitory factor (LIF)

Leukaemia inhibitory factor is a multi-functional cytokine which shares some properties with IL-6. It was originally described in the mouse following the induction of differentiation and the suppression of growth of the M1 mouse myeloid leukaemia cell line (Hilton et al, 1988). The human homologue has been cloned (Gough et al, 1988) and rhLIF has been given to nonhuman primates where it has been shown to raise the platelet count and to induce the synthesis of acute phase proteins (Mayer et al, 1993). Further characterisation of LIF is awaited.

2.3.16. Negative haemopoietic regulators

Spectacular progress in the knowledge of stimulatory growth factors for haemopoietic cells has been made in recent years; the development of knowledge of inhibiting factors that affect haemopoietic cell proliferation has been much slower. Inhibiting factors are likely to be of physiological importance in maintaining stem and early progenitor cells in quiescent state and in opposing the actions of stimulatory factors on progenitor cells. The balance between stimulatory and inhibiting influences offers a mechanism whereby haemopoiesis can be regulated (Axelrad, 1990; Moore, 1991b; Wright & Pragnell, 1992). The properties of some known negative regulators are summarised in Table 4 and described in

more detail below. The stem cell inhibitor, MIP-1a, has been described previously (p 8).

Transforming growth factor - beta (TGF-ß)

TGF-ß is a potent inhibitor of progenitor cell proliferation which has been shown to inhibit the colony formation of CFU-GEMM, CFU-Mega, BFU-E, CFU-E and CFU-GM (Sing et al, 1988; Hino et al, 1988; Cashman et al, 1990) but its role as a physiological regulator in haemopoiesis remains unknown. It also inhibits IL2 dependent T cell activation and is induced in activated T-cells where it may act in a physiological down regulatory response (Kehrl et al, 1986). In contrast, TGF-β which is contained in platelet α-granules stimulates fibroblast proliferation and wound healing following platelet activation (Haworth, 1989).

Tumour necrosis factor (TNF-α) and lymphotoxin

These two cytokines are closely linked inside the HLA complex on chromosome 6 and act with identical functions through the same receptor. TNF-α is principally released from activated macrophages in response to invasive stimuli and along with IL-1 is an essential trigger and mediator of the host inflammatory response. TNFα has many functions (Brenner, 1988) including actions on endothelial cells and fibroblasts to induce the production of GM-CSF, M-CSF, G-CSF and IL-6. However in contrast to IL-1, TNFα is inhibitory to the growth of haemopoietic progenitor cells in vitro especially in combination with gamma-interferon (Broxmeyer et al, 1986),

Leukaemia associated inhibitor (LAI)

This was first described as a factor secreted by myeloid leukaemia cells which reversibly inhibits normal CFU-GM growth but not that of leukaemia cells. This could provide a selective growth advantage for leukaemia cells and may explain the characteristic granulocytopenia observed in acute myeloid leukaemia (Olofsson and Olsson 1980; Axelrad, 1990). It has since been demonstrated to be produced by normal cells but its physiological significance remains unknown.

Inhibin

Inhibin is a glycoprotein hormone produced by the Sertoli cells of the testis and the granulosa cells of the ovary that acts as a negative feed back regulator to inhibit excessive secretion of follicle stimulating hormone (FSH) from the anterior pituitary gland. Inhibin has also been found in vitro to suppress erythroid colony formation of BFU-E and CFU-E of human bone marrow but only when these progenitors have been stimulated to grow by the FSH releasing protein activin. The CSF activity of activin is mediated through accessory cells which are probably T cells or macrophages. Inhibin has no effects on IL-3, GM-CSF or Epo stimulated colony formation (Axelrad, 1990). The physiological significance of this regulatory system of erythroid progenitor growth is unknown.

Negative regulatory protein (NRP)

NRP is a physiological negative regulator of murine erythropoiesis. It reversibly inhibits the stimulatory action of IL-3 on murine BFU-E (Axelrad, 1990). Its relevance to human haemopoiesis remains unknown.

Ac-SDKP tetrapeptide

Ac-SDKP is a tetrapeptide that is believed to be part of a larger molecule that is active in vivo. This molecule prevents the entry of murine CFU-S into cell cycle after sublethal irradiation or cytotoxic treatment and has been shown to protect mice from the lethal haematological side effects of the S-phase specific cytotoxic reagent cytosine arabinoside (Ara-C). The agent did not affect the response of the murine tumour to Ara-C. The discovery of a factor in humans which could reversibly protect human PHSCs would have significant therapeutic potential (Axelrad, 1990; Dexter and White, 1990).

Table 4. **Biochemical and functional properties of candidate negative regulators of human haemopoiesis[1].**

Name	Main cellular source	Chromosomal location	MW(Kd)	Principal Actions
MIP - 1α	macrophages bone marrow	unknown	50 - 100	inhibitor of murine CFU-S
TGF - β	T cells platelets bone marrow/ fetal liver	19q13	25 (homo dimer)	potent inhibitor of myeloid progenitors
TNFα and lymphotoxin	macrophages T cells, B cells, NK cells	6p21.3	17,15.5	trigger of host response to injury inhibitor of myeloid progenitors.
LAI	myeloid leukaemia cells marrow conditioned medium	unknown	125	inhibitor of CFU-GM
Inhibin	Testis, ovary	unknown	32(heterodimer)	inhibitor of BFU-E, CFU-E
NRP	murine marrow	unknown	79	inhibitor of murine BFU-E
AcSDKP	murine marrow	unknown	0.487	prevent entry of murine CFU-S into cell cycle.

[1] for more detailed explanation see text.

2.4 The Bone Marrow Microenvironment

The bone marrow is the only site in normal adult mammals where haemopoiesis takes place and following the injection of haemopoietic stem cells in experimental or clinical bone marrow transplantation procedures, long term haemopoiesis still establishes itself only in the bone marrow. These local tissue influences, critical for haemopoiesis, have collectively been named the bone marrow or haemopoietic micro environment. Immunologists, who showed that the thymus and the Bursa of Fabricius (in birds) were essential for T and B cell development respectively, highlighted first the importance of the micro environment in blood cell development but early data on the importance of stromal elements in the regulation of haemopoiesis came from the study of mice with mutational defects of the stromal cell environment. Such Sl mutant mice (Sl/Sl^d) exhibited defective erythropoiesis that could not be rescued by normal marrow stem cells and, when irradiated, such mice could not support the growth of transplanted normal CFU-S (McCulloch et al, 1965). However when bone marrow from normal mice was joined to Sl/Sl^d spleen, the implanted normal marrow stroma was able to support active erythropoiesis while adjoining Sl/Sl^d stroma remained quiescent (Wolf, 1979). The Sl mutation has now been characterised as a defect in the stromal production of stem cell factor (p 25).

Our further knowledge of the bone marrow microenvironment has been greatly increased following the development of techniques which have successfully reproduced in vitro the stromal cell dependent maintenance of sustained haemopoiesis. Early systems described culture conditions which were optimal for myelopoiesis (Dexter et al, 1977; Moore et al, 1979; Gartner and Kaplan 1980) but these were further developed to permit long term in vitro lymphopoiesis (Whitlock and Witte, 1982). In such long term bone marrow cultures (LTBMC) a heterogeneous, multi-layered adherent layer of stromal cells develops after 2-3 weeks of culture and in these cultures both putative PHSCs and haemopoietic progenitor cells can be maintained for up to several months. However, if the adherent layer fails to form or if it is disrupted, sustained haemopoiesis will not occur. This supportive bone marrow stroma contains an extra-cellular matrix (ECM) and a variety of cell types

including fibroblasts, endothelial cells, adipocytes and macrophages. Accessory cells such as T cells or monocytes/macrophages which may secrete multiple cytokines combine with the bone marrow stroma to complete the bone marrow microenvironment.

Haemopoietic precursor cells interact with the bone marrow microenvironment via the release of soluble cytokines and via a complex pattern of cell-cell and cell-matrix interactions (Torok-Storb, 1988; Gordon et al, 1988; Clark et al, 1992) to ultimately generate more mature cells that are then released from the adherent stromal layer. (Figure 7). For example, haemopoietic progenitor cells have been shown to be preferentially located in the adherent layer of LTBMC (Coulombel et al, 1983) and the colony-forming ability of the CFU-blast (believed to be a very primitive multipotent cell) described by Gordon et al, 1987 is stroma dependent. Developing granulocytes in LTBMC are usually found in association with adipocyte rich areas of stroma and maturing normoblasts and reticulocytes both in vivo and in vitro are commonly seen in close juxta-position to central macrophages (Dexter, 1982).

The cell-matrix interactions have been further characterised. The extra cellular matrix consists of fibronectin, laminin, multiple types of collagen and proteoglycans molecules (Gordon, 1988). Proteoglycans molecules consist of a protein core to which glycosaminoglycans molecules (GAG) such as chondroitin sulphate and heparin sulphate are attached. Studies have demonstrated that GAG can bind CSFs such as IL-3 and GM-CSF and present them to haemopoietic progenitor cells (Gordon et al, 1987b; Roberts et al, 1988) and have also shown that fibroblast derived fibronectin preferentially binds to early erythroid progenitors (Tsai et al, 1987). In addition to these cell-cell and cell-matrix interactions it is becoming increasingly recognised that many of the regulatory cytokines previously described can be produced within the bone marrow microenvironment (Dorshkind, 1990).

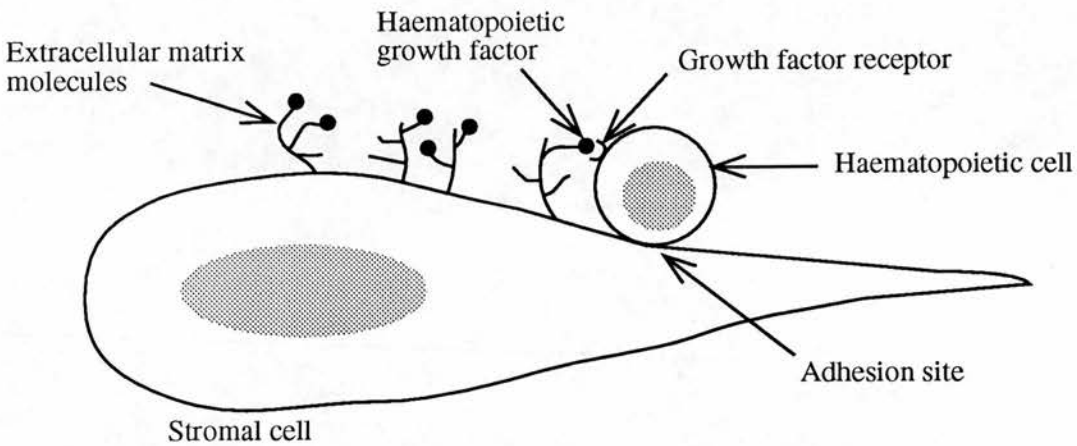


Figure 7: Schematic diagram showing the cellular and cell/matrix interactions operative within the bone marrow microenvironment. A complex network of interactions is generated by the 1) heterogeneity of the stromal cell and haematopoietic cell types; 2) production of several different growth factors and inhibitors; 3) heterogeneity of extra cellular matrix molecules and 4) differing processes which govern the adhesion of haematopoietic cells and CSFs to stromal constituents.

CHAPTER 3
INTRACELLULAR EVENTS ASSOCIATED WITH THE REGULATION OF NORMAL HAEMOPOIESIS.

Knowledge at the biochemical and molecular level of the intracellular events associated with the regulation of normal haemopoiesis is fundamentally important to our understanding of the mechanisms involved in disordered haemopoiesis. Previous studies have indicated that malignant haemopoietic cells exhibit cell surface characteristics similar to normal cells and that the mechanisms involved in their malignant potential relate to disordered internal events at the molecular level. Haemopoietic malignancies and dysplasias reflect an imbalance between the processes which integrate normal cell growth, lineage commitment and differentiation, illustrating how closely linked these processes are in both normal and abnormal cells. However, in contrast to our knowledge of the external factors that regulate haemopoiesis (See Chapter 2) our understanding of the internal molecular events associated with the processes of lineage commitment, cell growth and differentiation is extremely limited.

However a great stimulus to research in this area has been the identification of oncogenes and the recent cloning of haemopoietic growth factors and their receptors. Cellular proto-oncogenes (c-oncs) code for proteins which control cell growth and differentiation in normal cells. These products were first recognised in RNA tumour viruses as being the constituents (viral oncogenes) responsible for their ability to transform cells to a malignant phenotype. Study of the structure and sequence of viral oncogenes (v-oncs) has shown them to be closely homologous with cellular proto-oncogenes and it is believed therefore, that the viral genes evolved through retrovirus capture and subsequent mutation of cellular proto-oncogenes. It has since been shown that these cellular proto-oncogenes encode for products such as growth factors, growth factor receptors and mediators of intracellular transduction pathways. Abnormal cellular proto-oncogene expression is involved in human malignancies, including those of the haemopoietic system. The intracellular events that are involved in these processes are described in more detail below. To date the activities of known

transforming oncogenes and haemopoietic growth factors, are best understood in the context of mitogenesis and cell proliferation. In contrast, the intracellular events governing processes such as lineage commitment and cellular differentiation are poorly understood and the purpose of the experimental work described in this thesis is to develop our knowledge in these important areas.

3.1 Haemopoietic regulatory cytokines

The importance of haemopoietic growth factors to the survival, proliferation, differentiation and activation of haemopoietic cells at various stages of development has been described previously (Chapter 2). Of enormous additional significance to the biology of neoplasia was the discovery that the viral oncogene (v-sis) product was closely homologous with a human growth factor, the ß-chain of platelet derived growth factor (PDGF) (Waterfield et al, 1983). However the factors governing the gene expression of haemopoietic growth factors and the role of abnormal expression of CSFs in haemopoietic malignancy remain poorly understood. Although evidence has suggested that such cytokines as GM-CSF and IL-6 may play a role by either autocrine or paracrine mechanisms in the pathogenesis of acute myeloid leukaemia and myeloma respectively the intracellular mechanisms involved remain unknown (Lang and Burgess, 1990). What is clear, however, is that the genes that encode for certain haemopoietic growth factor receptors do have oncogenic potential (see below).

3.2 Haemopoietic regulatory cytokine receptors

The very recent cloning of haemopoietic growth factors and their receptors has facilitated further study of the intracellular biochemical events associated with CSF-induced cell proliferation and differentiation. It was the establishment of growth factor dependent cell lines which express high numbers of individual cytokine receptors that enabled a large number of cytokine receptors to be isolated, cloned and sequenced. This knowledge of the structure of cytokine receptors has generated increased understanding of the processes of receptor-ligand

interaction and subsequent intracellular signal transduction (Kaczmarski and Mufti, 1991; Vairo and Hamilton, 1991; Kan et al, 1992).

All cytokine receptors are transmembrane glycoproteins made up of an extracellular amino-terminal portion, responsible for binding the appropriate ligand, a short hydrophobic transmembrane region and a carboxy-terminal intracellular portion. The intracellular portion of the molecule is responsible for the transduction of signals that are the consequence of ligand binding. Many receptors share structural similarities and these have been grouped together in the cytokine receptor superfamily (Figure 8). This structural homology is also shared between haemopoietic and non-haemopoietic growth factor receptors (e.g. for growth hormone and prolactin) and may in part explain the overlapping actions of different cytokines and further supports the concept that the cytokine network has developed from a common evolutionary ancestor. The basis of this structural homology is a 210 amino acid stretch of the ligand binding domain which contains four conserved cysteine residues and a repeated tryptophan-serine motif separated by a random amino acid residue (Trp-Ser-X-Trp-Ser) which in most receptors is placed just proximal to the transmembrane domain (Figure 9). However, not all cytokine receptors share this particular structure. Other receptors, such as the M-CSF receptor, the IL-1 receptor and the PDGF receptor have immunoglobulin domains in their extracellular portions and form part of the immunoglobulin like superfamily. These receptors do not share the homologous regions of the cytokine receptor superfamily. There is however some overlap between the cytokine receptor and immunoglobulin superfamilies as illustrated by the receptors for IL-6 and G-CSF, both of which have immunoglobulin like domains and also structural homology with the cytokine receptor superfamily (Figure 8). The G-CSF receptor differs further by containing three fibronectin Type III domains between the region containing the conserved cysteines and the transmembrane domain (Figure 8). As fibronectin has been implicated in cell/cell interactions and heparin binding it is possible that this region is involved in the association of myeloid progenitor cells with marrow stromal elements and heparin sulphate-bound growth factors. There is little amino acid sequence homology in the intra-cytoplasmic portions of the

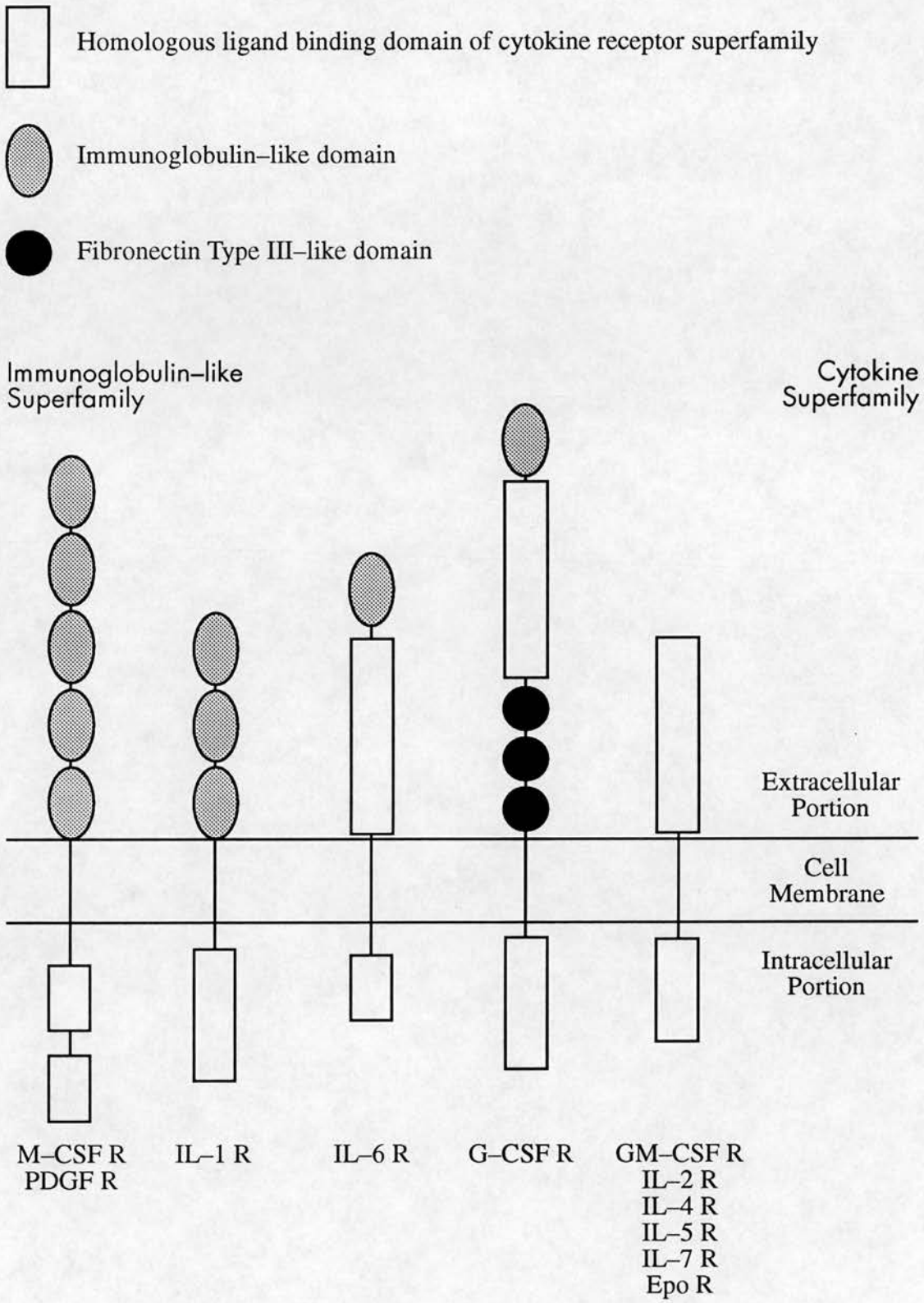


Figure 8: The overlap in structure between the immunoglobulin–like superfamily of receptors and the cytokine receptor superfamily

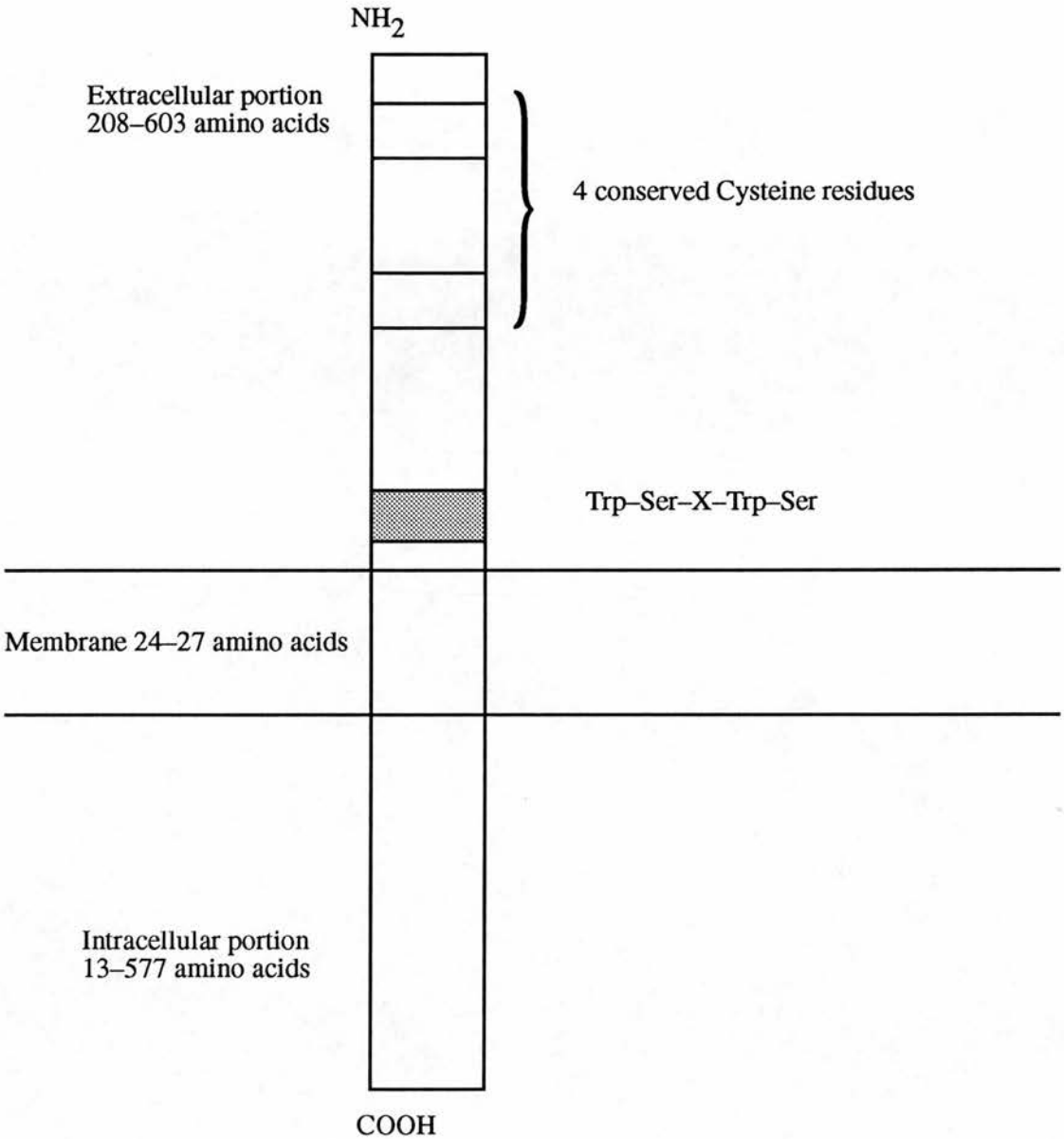


Figure 9: The generic structure of the cytokine receptor superfamily

cytokine receptor superfamily, but these intracytoplasmic portions are rich in proline and serine residues. The mechanisms involved in signal transduction at these receptors remain unknown. However, the receptors for M-CSF and PDGF (part of the immunoglobulin superfamily) and also the receptor for SCF, which is encoded by the c-kit oncogene, are known to effect signal transduction by intrinsic tyrosine kinase activity. The importance of protein phosphorylation in intracellular signalling is described below (p 44).

The importance of altered growth factor receptor expression in human malignancy has become increasingly recognised. The M-CSF receptor and the SCF receptor are encoded by the cellular proto-oncogenes c-fms and c-kit respectively. Point mutations within c-fms at codons 301 or 967, analogous to those found in v-fms have been found in vivo and may result in a constitutively active or upregulated receptor with resultant cell transformation (Tobal et al, 1990). Similarly mutations within the stem cell growth factor receptor gene (c-kit result in autonomous cell growth [Yarden, 1988; Catlett et al, 1990]) and the recent demonstration of a point mutation in the murine Epo receptor, which results in autonomous cell growth, suggests that the human EpoR is a potential cellular proto-oncogene (Yoshimura et al, 1990). The genetic mechanisms involved in the regulation of the expression of the high affinity alpha subunit component of the IL-2 receptor (IL-2α R) have been studied in detail and have provided insights into the pathogenesis of the HTLV-1 associated adult T-cell leukaemia/lymphoma syndrome (Kaczmarski and Mufti, 1991). HTLV-1 encodes a 40 kD trans-activating regulatory protein, Tax, that upregulates viral replication through binding to a DNA-binding site within the viral long term repeat (LTR) sequences. The viral DNA-binding site is homologous to the promoter regions for human IL-2αR and IL-2. The binding of Tax proteins to these sites sets up an autocrine loop enabling IL-2 secretion as well as IL2αR expression with resulting T-cell proliferation.

3.3 Intracellular signal transduction

The activation of a membrane receptor by the binding of a ligand such as a colony-stimulating factor generates an intracellular secondary

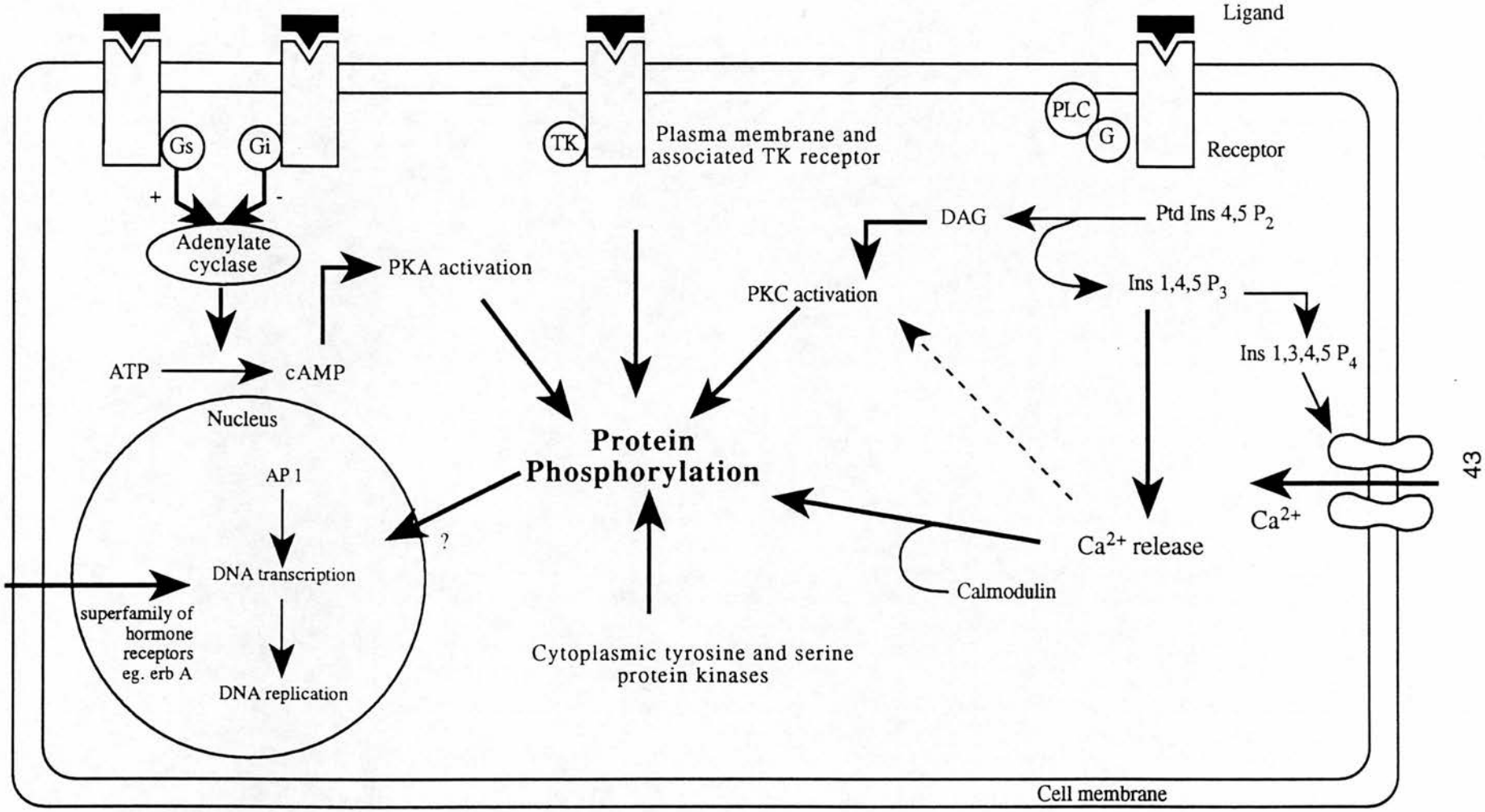


Figure 10: Potential signal transduction pathways involved in cellular proliferation

message that is transmitted to the nucleus to effect gene transcription and subsequent events such as DNA synthesis or cellular differentiation. Further study has shown that these biochemical messages involve changes in the intracellular metabolism of inositol and changes in the phosphorylation status of intracellular proteins. In some instances it has been shown that these phosphorylation events induce the expression of nuclear transcription factors that directly result in DNA synthesis. These intracellular signal transduction mechanisms are outlined in more detail below.

3.3.1. Intracellular protein phosphorylation

The discovery of cyclic adenosine monophosphate (c-AMP) dependent protein kinase some 20 years ago indicated how important reversible phosphorylation is in the regulation of protein function in eukaryotic cells (Cohen, 1982). Four groups of protein kinases which are involved in growth regulation have been described.

The c-AMP dependent protein kinase (PKA)

Certain ligand-receptor interactions particularly those involving hormones result in the activation of membrane bound adenylate cyclase to yield c-AMP as a second messenger. The interaction between the receptor and the second messenger system is mediated by regulatory guanine nucleotide binding (G) proteins that can either be stimulatory (Gs) or inhibiting (Gi) (Figure 10). The activation of PKA by c-AMP results in the phosphorylation of certain target proteins but the potential significance of these events in the control of cellular proliferation and differentiation remains unknown.

Phospho-inositol 4,5 bisphosphate hydrolysis and Protein kinase C

Receptor ligand interactions may result in the activation of phospholipase C mediated hydrolysis of the lipid phospho-inositol 4,5 bisphosphate (PIP_2) to release inositol 1,4,5 tris-phosphate (Ins (1,4,5)P_3) and 1,2 - diacyl glycerol(DAG) (Figure 10). Once generated in this way Ins (1,4,5) P_3 mediates the release of calcium from internal

stores which may activate calcium/calmodulin dependent protein kinases (see below) and may be further phosphorylated to produce inositol 1,3,4,5 tetrakisphosphate (Ins (1,3,4,5)P_4) which stimulates the transport of calcium from the extracellular environment into the cell. The second product of PIP_2 hydrolysis, DAG, remains within the membrane and activates the calcium and phospholipid dependent serine and threonine-specific protein kinase C (Nishizuka, 1988; Berridge and Irvine, 1989). Protein kinase C appears to have a central role in the regulation of cell activity. In particular PKC mediated phosphorylation events are believed to be important in receptor down regulation and both cellular proliferation and differentiation. Key regulatory molecules that are phosphorylated by PKC include the receptors for transferrin, insulin, T cell antigens and epidermal growth factor (EGF) and in the case of EGF receptor, phosphorylation results in inhibition of the receptor's own intrinsic protein kinase activity (Rayter et al, 1989). This finding reveals the intricate interactions of various protein kinases in the cell whereby apparently simple protein modifications can bring about marked changes in the physiology of cells. The tumour-promoting phorbol ester, phorbol myristate acetate (PMA) has been shown to directly activate PKC and PMA-treated cells have been shown to exhibit increased expression of the mitogenic proto-oncogene transcription factors c-jun, c-fos and c-myc.

These observations indicate that PKC activity may be an important regulatory pathway for the control of both normal and abnormal cellular proliferation. However PKC activation may also have an important role in growth arrest and cellular differentiation as PMA treatment of the human promyeloid cell line HL60 has been shown to promote terminal monocyte differentiation in these cells.

Calcium/calmodulin dependent protein kinases

Calmodulin is an intracellular protein that undergoes conformational change following binding with calcium which potentiates its capacity to activate various protein kinases. One such kinase, protein kinase III, phosphorylates elongation factor-2 (EF-2) which results in the dissociation of EF-2 from the ribosomes, thereby inactivating protein

synthesis. It has been proposed that rapid phosphorylation/ dephosphorylation of EF-2 in response to exogenous growth signals may allow newly synthesised RNAs produced shortly after mitogen binding rapid access to the translation machinery (Rayter et al, 1989).

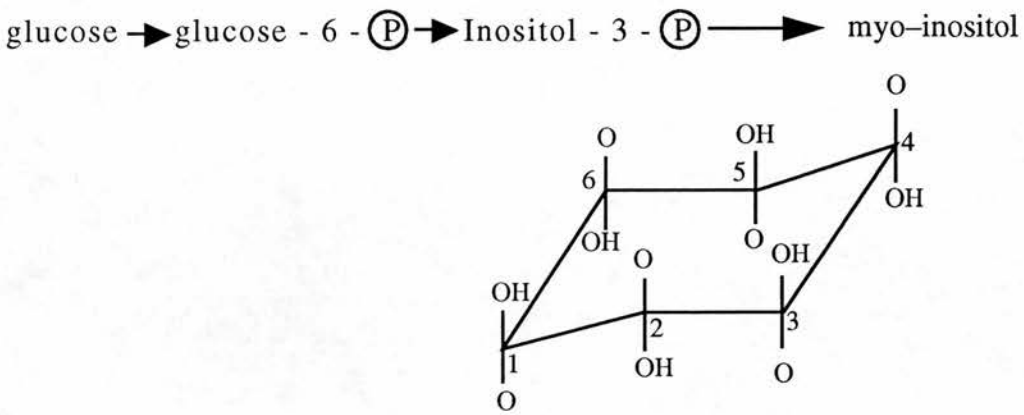
Protein tyrosine kinases

The observations that the membrane bound receptors for the growth factors SCF, M-CSF, EGF and PDGF (the first three of which are encoded by the cellular proto-oncogenes c-kit, c-fms and erb-B respectively) all have intrinsic tyrosine kinase activity in their cytoplasmic domains strongly support the concept that intracellular phosphorylation events are involved in their signal transduction pathways. Other cellular proto-oncogene products e.g. c-abl, c-src encode for cytoplasmic tyrosine kinases and the c-raf and c-mos proto-oncogene products encode for cytoplasmic serine/threonine protein kinases (Marshall, 1989). The substrates for these protein kinases remain unknown but oncogenic alterations in these products appear to result in elevated enzyme activity. For example the v-abl protein and the bcr-c-abl fusion protein, which is produced in most cases of chronic granulocytic leukaemia, have much greater tyrosine kinase activity than the native c-abl proto-oncogene product.

3.3.2. Nuclear transcription factors

The localisation of several cellular proto-oncogene products such as those for c-jun, c-fos, c-myc, p53 and erb-A, to the cell nucleus completed the link in the signal transduction pathway from the cell membrane to the nucleus. These proto-oncogene products are involved in the regulation of gene transcription and DNA replication. The c-jun proto-oncogene product encodes for the transcription regulator AP-1 which activates the transcription of several genes, including those for c-fos and c-myc, which are involved in cellular proliferation. The expression of genes which appear to be regulated by AP-1 can also be induced by the pre-treatment of cells with the phorbol ester, PMA, which is an activator of PKC. Therefore, this mechanism illustrates that a membrane initiated intracellular phosphorylation event can be

a) Synthesis of myo–inositol from glucose



b) Synthesis of 3 phosphatidyl inositol

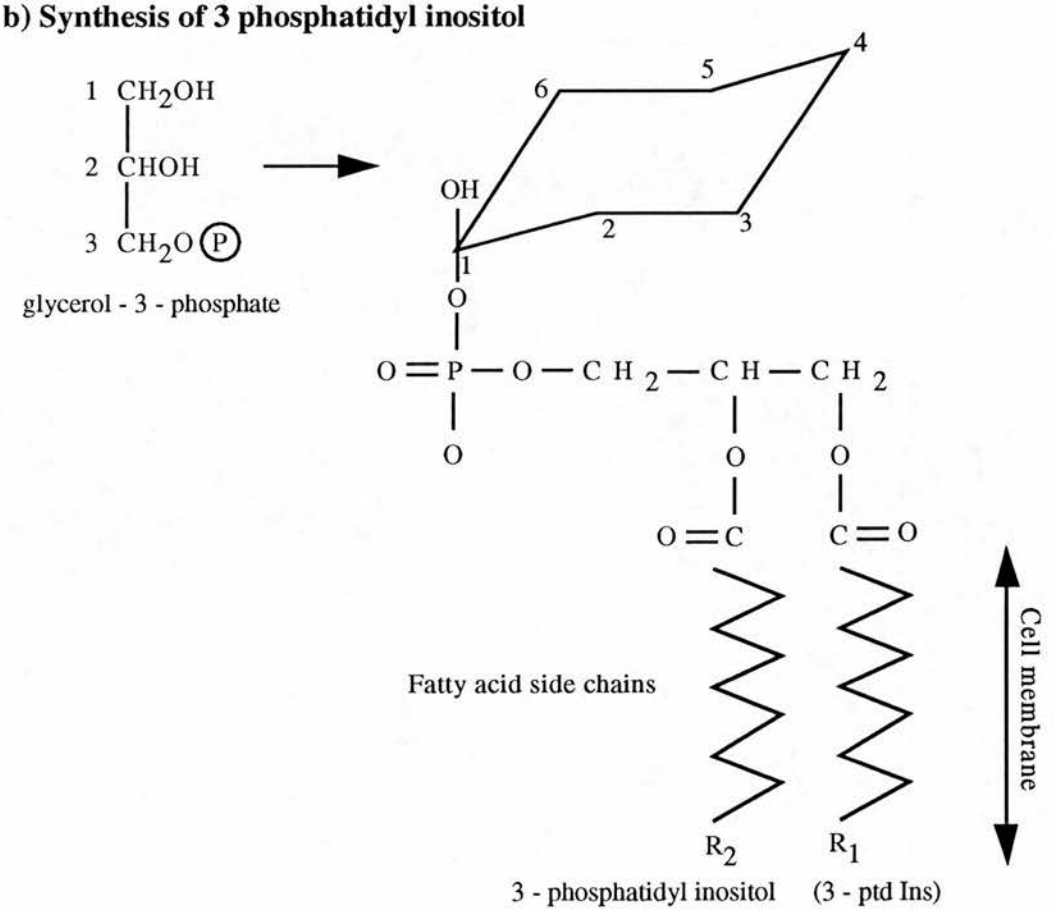


Figure 11: Basic structure and synthesis of myo–inositol and phosphatidyl–inositol (Ptd Ins).

For more detailed explanation see text.

intimately linked with DNA transcription (Rayter et al, 1989). The proto-oncogene c-erb A is the receptor for the hormone tri-iodothyronine (T_3). This is one of a superfamily of intracellular hormone receptors which are capable of binding to DNA and regulating gene transcription.

3.3.3. Inositol metabolism

Inositol is a cyclic six carbon sugar alcohol synthesised from glucose which exists in the biologically active stereoisomer form of myo-inositol (Figure 11a). The inositol ring forms an integral part of membrane bound phospholipids and also exists in water soluble intracellular inositol metabolites including glycerophospho-inositol (GPI), inositol and inositol phosphorylated at one or more positions of the inositol ring giving rise to inositol mono, bis, tris, tetra, penta and hexaphosphates. Evidence is mounting that several of the metabolites of inositol play important roles in the regulation of eukaryotic cells.

Inositol containing phospholipids

Inositol containing phospholipids, which are a ubiquitous component of all eukaryotic cell membranes, are based on the structure of phosphatidyl inositol (Ptd Ins, Figure 11b). Ptd Ins is based on a backbone of glycerol-3-phosphate which is produced by the glycolytic pathway. The hydroxyl (OH) groups of carbon atoms 1 and 2 of glycerol-3-phosphate are esterified by fatty acid side chains (R1, R2) which reside within the membrane lipid bilayer. The OH group of carbon number 3 is esterified with a phosphate group which is then attached to the 1 carbon position of myo-inositol. The OH groups of the different carbon atoms of the myo-inositol ring can be phosphorylated to yield various Ptd Ins phosphates. Ptd Ins (4) phosphate and Ptd Ins (4,5) bisphosphate are synthesised sequentially by the action of Ptd Ins 4-kinase and Ptd Ins (4)P-5 kinase respectively. Ptd Ins (4,5) bisphosphate (Ptd Ins (4,5) P_2) is the membrane bound precursor for the secondary messengers Ins (1,4,5) P_3 and Ins (1,3,4,5) P_4 described previously (p 44).

Another major role for inositol containing phospholipids is as a membrane anchor for surface proteins. Many proteins which do not contain hydrophobic membrane spanning sequences are covalently attached to phospholipids contained within the cell membrane. In these circumstances the carboxy terminus of the protein is linked via an ethanolamine/glucosyl linkage to the C6 position of phosphatidyl inositol. Because of the glycosyl-phosphatidyl-inositol linkage these proteins are known as GPI-linked proteins (Ferguson and Williams, 1988). Of additional interest in this area has been the recent discovery that the underlying defect in paroxysmal nocturnal haemoglobinuria (PNH) may be due to the defective linkage of GPI linked proteins in affected cells. PNH is associated with protean clinical manifestations which are caused by the absence of important regulatory molecules from the surface of haemopoietic cells. These molecules include complement control proteins such as decay accelerating factor, membrane inhibitor of reactive lysis (MIRL, CD59) and C8 binding protein which, when reduced on PNH erythrocytes, increase the susceptibility of these cells to complement mediated lysis. Other missing surface proteins include lymphocyte function antigen 3 (LFA-3, CD 58) and the major receptor on neutrophils for the Fc portion of IgG (FCRIII, CD16). Absent LFA-3 may contribute to the defective control of haemopoiesis observed in PNH and the absence of FCRIII from neutrophils may contribute to the increased susceptibility to infection seen in this condition. The common feature of these and the other surface proteins missing in PNH is that they are all attached to the cell membrane by GPI anchors (Rosse, 1990).

Intracellular inositol phosphates

In addition to lipid containing inositol phosphates, eukaryotic cells also contain phosphorylated forms of myo-inositol. These compounds, being highly polar, are water soluble and are predominantly located within the cytoplasm. Detailed information concerning the metabolism and functions of intracellular inositol metabolites, particularly, the highly phosphorylated forms, is lacking but recent evidence suggests that they may have a role in myeloid differentiation (Michell et al, 1990). Cells from the human promyeloid cell line HL60 contain high concentrations

of the highly phosphorylated inositol compounds, inositol pentakisphosphate (Ins P_5) and inositol hexakisphosphate Ins P_6) (French et al, 1991). During dimethylsulphoxide (DMSO) induced neutrophil differentiation of HL60 cells, there is a 3-fold increase in the intracellular concentration of Ins P_5 and, in contrast, following PMA-induced monocyte differentiation the intracellular concentration of Ins P_5 in HL60 cells falls by 90% (French et al, 1991). In addition to a fall in Ins P_5 PMA-induced monocyte differentiation was also associated with a fall in the intracellular concentrations of inositol tetrakisphosphate (Ins P_4) and Ins P_6. These changes, which may suggest an important role for the abundant inositol polyphosphates in myeloid differentiation, were investigated further in this thesis. It was important to determine whether the high levels of the highly phosphorylated inositol compounds seen in HL60 cells are a feature of either leukaemic potential or immature myeloid cells and also whether the observed changes in levels seen in HL60 cells also occur in normal myeloid cells as they undergo differentiation. To study these questions a homogeneous population of undifferentiated myeloid blast cells was purified from human fetal liver and subjected to analysis of the intracellular concentrations of inositol phosphates as they underwent PMA-induced monocyte differentiation.

CHAPTER 4.
MODEL SYSTEMS FOR THE STUDY OF INTERNAL BIOCHEMICAL EVENTS IN HUMAN HAEMOPOIETIC CELLS.

The opportunity to study internal biochemical and molecular events within haemopoietic cells, which are related to lineage commitment, proliferation and differentiation is dependent upon the availability of suitable experimental model systems. Although considerable progress has been made from the study of murine experimental systems, these may not be relevant to human haemopoiesis, particularly where disordered events result in lineage aplasias, dysplasias and neoplasias. The relative advantages and disadvantages to be gained from studying differing models of human haemopoiesis are presented below. It is proposed that an integrated approach, gaining knowledge from all available model systems, be followed in order to resolve these issues.

4.1. Haemopoietic cell lines

Haemopoietic cell lines offer considerable advantages through their ability to produce large numbers of cells which are relatively homogeneous and routinely available. The numbers of cells available are sufficient for biochemical and molecular studies (Lubbert and Koeffler, 1988). Some examples of haemopoietic cell lines which have been particularly useful as models for the study of haemopoietic cell proliferation and differentiation are listed in Table 5. Other haemopoietic cell lines have also made significant contributions. Because many cell lines are dependent on growth factors for either their survival or proliferation potential, such cell lines have been used 1) in bioassays for cytokines (e.g. for IL-2 or for IL-6), 2) for the isolation and purification of high affinity cytokine receptors and 3) for the study of the mechanisms of cytokine signal transduction and subsequent cytokine actions.

However significant differences exist between normal cells and cell lines. Normal haemopoietic cells are factor dependent and have normal karyotypes. Tumour cells are also generally factor dependent and have subtle genetic differences which may or may not be detectable at the karyotypic level. Haemopoietic cell lines have been generated from

tumour cells and these cell lines generally lose their factor dependence and also become increasingly genetically altered with continued passage in tissue culture. Therefore cell lines have significant limitations because they neither represent normal cells nor de novo leukaemia cells which are the cell populations of greatest interest.

Therefore disadvantages of cell lines as model systems include the limited choice of suitable cell lines available for study and problems concerning the interpretation of the results obtained. For example it may be difficult to determine whether the effects observed relate to events occurring 1) as part of normal cellular processes, or 2) whether the events are related to the cells abnormal transformed status or 3) relate to long term passage in vitro. Furthermore the limited range of haemopoietic cell lines available already show evidence of lineage commitment and are therefore not amenable for the study of events relating to the lineage commitment, proliferation and differentiation of the most primitive haemopoietic precursors.

An interesting model system not yet available for the study of human haemopoiesis has been the isolation of a series of murine multi-potential haemopoietic cell lines known as FDCP-Mix which grow continuously in the presence of IL-3 (Factor-Dependent Continuous cell lines, Paterson Laboratories, Spooncer et al, 1986). These cells are non-leukaemic and have a normal karyotype and in the absence of IL-3 they die. However when cultured in association with marrow stromal cells in the absence of IL-3 or in low concentrations of IL-3 in combination with other myeloid growth factors, these cells show evidence of differentiation along different myeloid lineages (Heyworth et al, 1990). It would be a great advantage to isolate from humans similar normal multi-potential growth factor dependent haemopoietic continuous cell lines.

4.2. Normal human haemopoietic cell populations

To overcome the problems of interpretation of results obtained from the study of transformed cell lines one would prefer to study normal cell populations. However, for the study of biochemical and molecular events, one needs to obtain large numbers of cells as a homogeneous

Table 5. **Human haemopoietic cell lines which have been used as model systems for the study of haemopoietic proliferation and differentiation.**

Cell Line	Differentiation Status	Model Uses	Reference
KG1a	early myeloblast	production of CD34 McAb	Koeffler et al, 1980
K562	myeloblast/erythroblast	megakaryocyte/erythroid differentiation	Lozzio and Lozzio, 1975 Alitalo, 1990.
HL60	myeloblast/promyelocyte	cell kinetics granulocyte/monocyte differentiation	Collins et al, 1977
U937	monoblast	monocyte differentiation/function	Sundstrom and Nilsson, 1976
RS4;11	pre-B cell/monoblast	B lymphoid/monocyte differentiation	Strong et al, 1985.

population but it has proved difficult to isolate haemopoietic precursor cells in sufficient numbers for such studies. Haemopoietic precursor cells only exist at very low frequencies in haemopoietic tissues such as adult bone marrow, fetal liver and neonatal cord blood. However, the increasing sophistication of haemopoietic precursor cell fractionation procedures and the ability to maintain undifferentiated cells in serum-free media in the presence of cocktails of the recently cloned haemopoietic growth factors have permitted a great opportunity to generate homogeneous populations of normal haemopoietic precursors in sufficient numbers for appropriate biochemical studies. The cell fractionation techniques employed have included density gradient separation, separation of cells following lectin or plastic adherence, positive or negative selection using monoclonal antibodies directed against cell surface antigens, cell sorting following flow cytometry, cell separation using centrifugal counter flow elutriation and the treatment of cells by S phase specific chemotherapeutic agents. The studies described in this thesis include the development of a homogeneous population of undifferentiated myeloid cells from human fetal liver which was used for subsequent biochemical analysis.

However the availability of model systems for biochemical and molecular analysis is of limited value unless one knows what events to study. This is a problem when the events are unknown, such as those internal events which regulate lineage commitment, proliferation and differentiation within haemopoietic cells. Under these circumstances one either has to adopt a shotgun approach and study many avenues blindly in the hope that one will prove fruitful or alternatively select individual avenues for study based on previous observations made from other model systems. One major area where clues have arisen as to the mechanisms governing normal and abnormal cell development has come from the study of individual cases of haemopoietic lineage neoplasias and dysplasias.

4.3. Individual cases of haemopoietic neoplasias and dysplasias

Individual cases of leukaemias and lineage dysplasias result from perturbations of the normal cellular processes which control lineage

commitment, proliferation and differentiation. Many of these cases have been associated with structural chromosomal abnormalities such as chromosomal translocations, inversions, deletions and amplifications (Rabbitts and Rabbitts, 1989; Berger, 1992). Further study of these chromosomal abnormalities has indicated that genes which code for key regulators of lineage commitment, proliferation and differentiation are located at or near the sites of the chromosomal aberrations. In cases of chromosomal deletions, these abnormalities may be a guide to the location of tumour suppression genes and such abnormalities have been shown to be involved in the pathogenesis of retinoblastomas and Wilm's tumours (Rabbitts and Rabbitts, 1989). Examples where the increased expression or activation of known cellular proto-oncogenes occurs in human haemopoietic malignancies as a result of major chromosomal abnormalities are shown below in Table 6.

Of special relevance to the pathogenesis of the B lineage malignancies documented in Table 6 is the involvement of immunoglobulin (Ig) gene loci in the chromosomal aberrations. For example the Ig heavy gene locus is located at 14q32, the Ig kappa light chain gene locus is located at 2p12 and the Ig lambda chain gene locus is located at 22q11. Of parallel interest is the observation that many human T cell leukaemias are associated with abnormalities of chromosome 14 principally involving band 14q11, the location of the gene for the alpha-chain of the T cell receptor (TCR-α). The juxta position of oncogenes next to those for either the Ig or TCR genes may result in the aberrant expression of these oncogenes resulting in either B or T cell neoplasias (Rabbits and Rabbitts, 1989; Berger, 1992).

Acute promyelocytic leukaemia is another haemopoietic malignancy associated with a specific chromosomal abnormality [t(15;17)(q22;q21)] where a re-arranged gene has been implicated in the pathogenesis of the disease. In this case the gene for the alpha receptor for retinoic acid (RAR-α) is translocated to join a chromosome 15 transcription unit, originally named MYL but now called PML, and transcribed as part of a fusion mRNA which is believed to encode for an abnormal RAR-α protein. The normal RAR-α gene codes for a nuclear receptor protein that acts as a transcription enhancer involved in the regulation of

Table 6. **Known haemopoietic malignancies associated with abnormal cellular proto-oncogene expression[1].**

Malignancy	Proto-oncogene (Chromosomal location)	Proto-oncogene function	Chromosomal abnormality
CGL[2]	c-abl (9q34)	intracellular tyrosine kinase	t(9;22)(q34;q11)
Burkitt's lymphoma	c-myc (8q24)	DNA binding protein	t(8;14)(q24;q32) t(2;8)(p12;q24) t(8;22)(q24;q11)
Follicular lymphoma	bcl-2 (18q21)	cell survival signal	t(14;18)(q32;q21)
B cell - CLL[3]	bcl-1 (11q13) prad1	unknown cyclin	t(11;14)(q13;32)[4]

1. For more details see Rabbits and Rabbits, 1989.
2. CGL = chronic granulocytic leukaemia
3. CLL = chronic lymphocytic leukaemia
4. References Tsujimoto et al, 1984; Rosenberg et al, 1991.

myeloid differentiation (Biondi et al, 1991). It has recently been shown that these patients respond to differentiation therapy with all-trans retinoic acid (trans RA) and it is believed the arranged RAR-α gene may confer therapeutic sensitivity of the disease to trans RA (Warrell et al, 1991; Borrow & Solomon, 1992)).

However there are many well defined structural chromosomal abnormalities which occur in association with human haemopoietic malignancies which have not been resolved at the molecular level (Berger,1992). Some examples are listed below in Table 7. It is likely that key genes involved in haemopoietic cell regulation will be found to be located at the site involved in these chromosomal aberrations.

Table 7 Human haemopoietic malignancies associated with well described chromosomal abnormalities which have not been defined at the molecular level.

Malignancy	Chromosomal abnormality
AML (M2)	t(8;21)(q22;q22)
AML (M4Eo)	inv(16) (p13;q22) or del(16)(q22)
AML (M5)	t(9;11)(p21;q23)
ALL	t(4;11)(q21;q23)
ALL	t(1;19)(q23;p13)

AML Acute myeloblastic leukaemia

ALL Acute lymphoblastic leukaemia.

In addition to cytogenetic and molecular DNA analysis, cells from cases of haematological dysplasias and neoplasias can be used in culture studies. Indeed, this is how haemopoietic cell lines have been produced but only a very small minority of such cultures ultimately generate transformed phenotypes which can be maintained in culture indefinitely. The recent cloning of many haemopoietic growth factors and cytokines, on which both many normal and malignant haemopoietic cells are dependent for survival, has offered the opportunity to maintain such

cells in culture for prolonged periods in order that biochemical and molecular studies can be performed. Indeed, the option of studying both normal cells and their transformed counterparts enables comparative analyses to be undertaken which may indicate the molecular phenomena associated with neoplasia.

As the purification of adequate numbers of homogeneous normal haemopoietic precursors for studies of lineage commitment and differentiation has proved difficult, it is useful to consider the potential of abnormal stem cells for similar studies. In this regard cases of primary myelofibrosis (PMF) are of considerable interest as PMF is generally associated with greatly increased levels of circulating haemopoietic progenitors including multi-lineage progenitors (CFU-GEMM), megakaryocyte progenitors (CFU-Meg), erythroid progenitors (BFU-E) and granulocyte-macrophage progenitors (CFU-GM)(Partenen et al, 1982; Douer et al, 1983; Hibbin et al, 1984; Carlo-Stella et al, 1987; Craig et al, 1991). Indeed many cases of PMF have even shown erythropoietin independent erythroid colony formation (Hibbin et al, 1984; Carlo-Stella et al, 1987). Therefore, as part of this thesis a single patient with PMF was selected for investigation to assess the feasibility of using such patients as a source of haemopoietic stem cells suitable for further study. The results obtained were of considerable interest and they generated different avenues of further study which are outlined in the next chapter.

CHAPTER 5.
AIMS OF STUDY

The principal objectives of this study were:

1) to purify an undifferentiated normal myeloid blast population from human fetal liver that would be suitable for the study of intracellular biochemical events which occur during normal myeloid differentiation;

2) to study changes in the intracellular concentrations of inositol phosphates as these undifferentiated normal myeloid blast cells underwent phorbol myristate acetate-induced monocyte differentiation;

3) to compare the results obtained for values of intracellular concentrations of inositol phosphates within undifferentiated normal myeloid cells with those previously obtained from the study of HL60 cells and to compare the changes observed after PMA-induced monocyte differentiation of both cell types.

As a contributory part of this thesis an individual case of primary myelofibrosis was selected for further study for two reasons:

1) to determine the potential of such patients as a source of haemopoietic cells to develop model systems which can be used to characterise biochemical and molecular events associated with lineage commitment, proliferation and differentiation in the human haemopoietic system;

2) to confirm clinical and morphological features in the patient suggesting an intrinsic defect in erythropoiesis and by further karyotypic and molecular study to determine the possible chromosomal location of genes which encode key regulators of erythroid lineage development. The clinical, haematological and morphological features of the case are described in the material and methods section (p 61).

SECTION 2. MATERIALS AND METHODS

CHAPTER 6. CELLS

6.1 Normal Cells

Normal peripheral blood cells were used as control cells for histochemical and immunostaining procedures performed on fractionated fetal liver cells. They were also used as positive control cells for titrations of monoclonal antibodies used in this study.

6.1.1. Peripheral blood mononuclear cells

Mononuclear cells, consisting of lymphocytes and monocytes were prepared from the peripheral blood of healthy donors by ficoll fractionation (Boyum, 1968). Heparinised blood (8 mls) was layered onto 3 ml ficoll hypaque (Pharmacia, UK) and centrifuged at 400g for 40 minutes at 20°C. The interface cells were carefully harvested with a Pasteur pipette and washed in RPMI 1640 medium (Gibco, UK) containing 2% V/V fetal calf serum (FCS, Gibco, UK), first at 500g for 10 minutes, then twice at 250g for 10 minutes at 20°C.

6.1.2. Dextran sedimented peripheral blood leucocytes

Neutrophil rich suspensions were prepared by erythrocyte sedimentation of peripheral blood using a solution of 6% hydroxyethyl starch in 0.9% saline (Hespan, Du Pont, UK). Heparinised blood was mixed with hespan in a ratio of 7:1 and the mixture was incubated at 37°C for 60 minutes. The upper layer containing leucocytes was carefully removed and washed three times in RPMI 1640 medium containing 2% V/V FCS (250g for 10 minutes at 20°C).

6.1.3 Bone marrow cells

Bone marrow cells from haematologically normal donors were obtained, with informed consent and local hospital ethical committee approval, either from the sternum of patients undergoing cardiac surgery or from

donors for allogeneic bone marrow transplantation. Fresh bone marrow was collected into Iscove's Modified Dulbecco's Medium (IMDM, Gibco, UK) containing 2.5 iu/ml preservative free heparin (monoparin, CP Pharmaceuticals, UK). Mononuclear cells used for assays of colony forming cells were fractionated on ficoll hypaque as described above and washed three times in IMDM containing 2% V/V FCS. For the establishment of long term bone marrow culture stromal layers, dextran sedimented cells were prepared as described above and washed three times in IMDM containing 2% V/V FCS.

6.1.4. Tonsillar B lymphocytes

Tonsillar lymphocytes in the G_0 phase of cell cycle were used in comparative cell cycle studies. These were prepared by Dr J Gordon, Department of Immunology, from tonsillectomy specimens provided by the Ear, Nose and Throat Hospital, Birmingham. A single cell suspension was prepared by using fresh tonsils free of red cells and by teasing small pieces of lymphoid tissue in buffered balanced salt solution (BBSS). Lymphocytes were obtained by fractionation using ficoll hypaque, as described above. B cells were purified by negative selection after rosetting T cells with sheep erythrocytes and B cells in the G_0 phase of the cell cycle were further purified by using a 62.5% Percoll (Pharmacia, UK) gradient. Cells banding below 62.5% Percoll constituted the G_0 population of tonsil cells.

6.2. Cells from a patient with primary myelofibrosis

6.2.1. Patient's details

A 71 year old man presented with anaemic symptoms, 8cm hepatomegaly and 12cm splenomegaly. His Hb was 4.3g/dl, WBC 2.8 x 10^9/l with neutrophils 2.2 x 10^9/l including hypersegmented forms, lymphocytes 0.3 x 10^9/l, monocytes 0.2 x 10^9/l ,myelocytes <0.1 x 10^9/l , blasts 0.1 x 10^9/l, normoblasts 1 per 100 WBC and platelet count 95 x 10^9/l . His blood film showed tear drop poikilocytes but infrequent normoblasts which were rarely seen on subsequent blood films. His reticulocyte count was 33 x 10^9/l and both the serum and red

cell folate were reduced. Bone marrow aspiration was unsuccessful and a trephine biopsy was hypercellular showing marked granulocytic hyperplasia, absent normoblasts, megakaryocytes of increased size with irregular nuclei and increased reticulin fibrosis, consistent with features in cases reported by Barosi et al, 1983. He was initially supported with folic acid, allopurinol and blood transfusion and was subsequently treated with hydroxyurea and oxymethalone. He remained transfusion dependent but after 7 months developed an increasing leucocyte count with an increasing proportion of blast cells which were refractory to therapy with hydroxyurea. He died 9 months after presentation having followed a course similar to patients reported by Bentley et al, 1977.

6.2.2. Peripheral blood cells

Venous blood taken into dipotassium ethylenediaminetetra-acetic acid (K_2 EDTA) was used for full blood count and blood film analysis. These investigations were carried out by the Haematology department of the Queen Elizabeth Hospital (QEH), Birmingham. For cytogenetic analysis, assays of colony forming cells and cell culture studies, mononuclear cells separated from peripheral blood collected into preservative free heparin were used. These cells were fractionated on ficoll hypaque as described above and washed three times in IMDM.

6.2.3 Bone marrow trephine biopsy

A bone marrow trephine biopsy was taken from the patient's right posterior iliac crest under local anaesthesia and processed by standard techniques in the QEH Department of Pathology. In brief, the tissue was fixed and decalcified for 72 hours in 10% neutral buffered formalin solution containing at first 5% and then 10% glacial acetic acid. The tissue was then dehydrated in serial alcohol solutions, cleared through serial chloroform solutions and finally embedded in paraffin wax. Sections of 3μ thickness were cut, mounted onto glass slides and then stained with haematoxylin and eosin and also for reticulin fibres.

6.2.4 Skin Biopsy

A 2 cm elliptical skin biopsy was removed from the patient's forearm under local anaesthesia and placed in sterile RPMI 1640 medium. This tissue was used to generate primary fibroblast cultures which were used to assess the constitutive karyotype and to provide a source of constitutive DNA.

6.3. Cell Lines

The cell lines listed in Table 8 were used as positive control cells for antibody reagents used in the fractionation of cells from human fetal liver. The relevant membrane surface antigen and reactive antibody are listed under the cell type. The human promyeloid cell line HL60 (Gallagher et al, 1979) was used in comparative biochemical studies with myeloid blast cells fractionated from human fetal liver.

Table 8. Human haemopoietic cell lines used in the quality control of antibody reagents

CELL LINE	CELL TYPE	REFERENCE
K562	Early erythroid Glycophorin C positive reactive with McAb Ret40F	Lozzio and Lozzio, 1975
U937	Promonocyte Common leucocyte antigen positive Reactive with McAb BK19.45.	Sundstrom and Nilsson, 1976
KG1a	CD34 antigen positive Reactive with McAb ICH3.	Koeffler et al, 1980

The cell lines were grown in suspension in RPMI 1640 medium containing 10% V/V FCS and 100 U/ml penicillin and 100 μg/ml

streptomycin (P/S; Gibco, UK). The cultures were maintained in the exponential phase of growth by seeding in 80 cm^3 tissue culture flasks (Nunc, UK) at a density of 2-3 x 10^5/ml, feeding with fresh medium on days two or three and subculturing when the cells had reached a density of 1-2 x 10^6/ml. All cultures were maintained in a LEEC incubator at 37°C in a humidified atmosphere containing 5% CO_2. Cell lines were cryopreserved as follows. Cells from a healthy culture were pelleted and resuspended at 1 x 10^8 cells/ml in cold FCS containing 5% dimethylsulphoxide (DMSO, Fisons Scientific,UK). The cell suspension was aliquotted into a 1ml sterile freezing ampoule, placed in an expanded polystyrene container and incubated for 30 minutes at - 20°C, 6 hours at -70°C and then transferred into liquid nitrogen. This method was also used to cryopreserve peripheral blood mononuclear cells and skin fibroblasts obtained from the patient with primary myelofibrosis. To recover viable cells after storage in liquid nitrogen the following thawing procedure was adopted. Ampoules containing cells were thawed quickly at 37°C and the cells transferred to a universal container on ice. Twenty mls of cold RPMI 1640 containing 10% FCS were added dropwise to cells which were then subsequently washed twice, assessed for viability, and finally resuspended at 5 x 10^5/ml in the appropriate culture medium.

CHAPTER 7.
FRACTIONATION OF FETAL LIVER CELLS

7.1 Tissue

Whole fetal livers were obtained from abortuses from prostaglandin induced terminations of mid trimester pregnancies which had been carried out at two local hospitals, the Robert Nursing Home and the Calthorpe Nursing Home. All handling of fetal tissue was performed in agreement with a code of practice recommended by a Government Committee, the Polkinghorne report (Command Paper CM762 HMSO, 1989), and was supervised by a central co-ordinator, Professor P H Gallimore of the Department of Cancer Studies, University of Birmingham. Under these guidelines maternal consent for the use of fetal material for research purposes had been obtained by a neutral third party and the identity of patients and source of fetal material was unknown to the investigators. The research utilising fetal tissue was carried out under special licence from the Department of Health and Social Security and in addition was approved by the local hospital ethical committee.

Freshly delivered fetuses ranging from 16-20 weeks gestation were placed in refrigerated containers and were available for collection, 2-18 hours following delivery. Tissue viability was shown to be variable but was not obviously related to the interval following delivery of the fetus. However, storage of delivered fetuses at refrigerated temperatures was important and efforts were made to achieve this aim as much as possible. Individual fetuses were placed in sterile dissecting trays and washed externally with alcohol. Using sterile instruments, the fetal liver was removed intact and placed in sterile RPMI 1640 medium containing 100 U/ml penicillin and 100μg/ml streptomycin (P/S).

7.2 Preparation of mononuclear cells

During the course of the project mononuclear suspensions of fetal liver cells were obtained using two different methods. The first method had been developed previously in our laboratory (Toksoz and Brown, 1984).

This method was later superceded by a method incorporating enzyme digestion of liver tissue. Both methods incorporated ficoll hypaque fractionation of cell suspensions to harvest cells of buoyant density <1.077. Haemopoietic stem cells and progenitor cells are known to be present within this fraction of cells.

7.2.1 Preparation of mononuclear cells following mechanical disruption of tissue

Whole livers were washed three times in RPMI 1640 medium and placed in a sterile plastic Petri dish (Sterilin, UK) in 10 mls of medium. Connective tissue around the liver and the gall bladder were removed, and the liver gently teased with forceps and scissors. Fragments were initially disrupted by aspirating through a 10 ml syringe and then through a 19 gauge needle. The cell suspension was made up to 40 mls with RPMI 1640, transferred to two sterile universal containers and then filtered through sterile cotton wool three times. The cotton wool had been previously washed in de-ionised water and the cotton wool columns were prepared by loosely packing, under water, 1ml cotton wool into the barrel of a 5 ml syringe. The syringe barrels were then drained and autoclaved in small sealed storage packets. The resulting filtered single cell suspension was then fractionated by centrifugation over ficoll hypaque as previously described. The interface cells were carefully harvested with a sterile Pasteur pipette and washed three times, as described previously, in RPMI containing 100 U/ml penicillin, 100 μg/ml streptomycin and 5% V/V FCS. Viable nucleated cells, as assessed by phase contrast microsocopy, were enumerated in a haemocytometer chamber. At least 200 cells were counted.

7.2.2. Preparation of mononuclear cells following enzyme digestion of tissue

This method is illustrated schematically in Figure 12. Following washing in medium and the removal of surrounding connective tissue as described above, the liver was gripped by forceps at the junction of the right and left main bile ducts as they emerged from the liver. Using a pair of scissors haemopoietic tissue was gently teased by blunt

dissection away from the connective tissue of the biliary tree which was then discarded. Larger tissue fragments were initially disrupted by aspiration through a 10 ml syringe and then by passage through a 19 gauge needle. The cell suspension was made up to 25 mls with RPMI 1640 medium and transferred to a 25 cm^2 tissue culture flask to which was added 5 mls of a freshly prepared enzyme solution containing 3 mg/ml hyaluronidase (Sigma, UK), 3 mg/ml collagenase (Sigma, UK) and 6 mg/ml dispase (Sigma, UK). The enzymes had been dissolved in serum free RPMI 1640 medium and the solution sterilised by passage through a 0.2 μm disposable filter (Gilman Sciences, UK). The cell suspension was then incubated with the enzymes at 37°C for 45 minutes with gentle agitation at 15 minute intervals. This resulted in the dissolution of small tissue fragments into a unicellular suspension. After 45 minutes incubation the enzyme digest mixture was made up to 60 mls with RPMI 1640 medium containing 10% V/V FCS to inhibit further enzyme activity. After further passage through a 19 gauge needle an aliquot of 100 μl was removed for cell counting and cytocentrifuged preparations and the remainder dispersed in 5 x 12 ml volumes for fractionation on 5 x 6 ml cushions of ficoll hypaque in universal containers (400g for 40 minutes at 20°C). Ten mls of supernatant medium were removed before harvesting the total interface using a sterile Pasteur pipette. The cells were washed three times in RPMI 1640 containing 2% V/V FCS as previously described and were then resuspended in 24 mls RPMI 1640 medium containing 2% V/V FCS. An aliquot of 100 μl was removed for counting and cytocentrifuged preparations and the cells were fractionated a second time on ficoll hypaque in two 12 ml aliquots as described above. The interface cell population was harvested and washed as described above and an aliquot removed for cell counting and cytocentrifuged preparations. On some occasions, due to the availability of fetal tissue in the late evenings, cells were incubated overnight following enzyme digestion and prior to ficoll fractionation which was performed the following day. In these circumstances, following enzyme digestion, the cells were pelleted at 400 g for 10 minutes, washed once in RPMI 1640 containing 10% V/V FCS and resuspended in 60 mls RPMI 1640 medium containing 20% V/V FCS for overnight incubation at 37°C in a humidified atmosphere containing 5% CO_2.

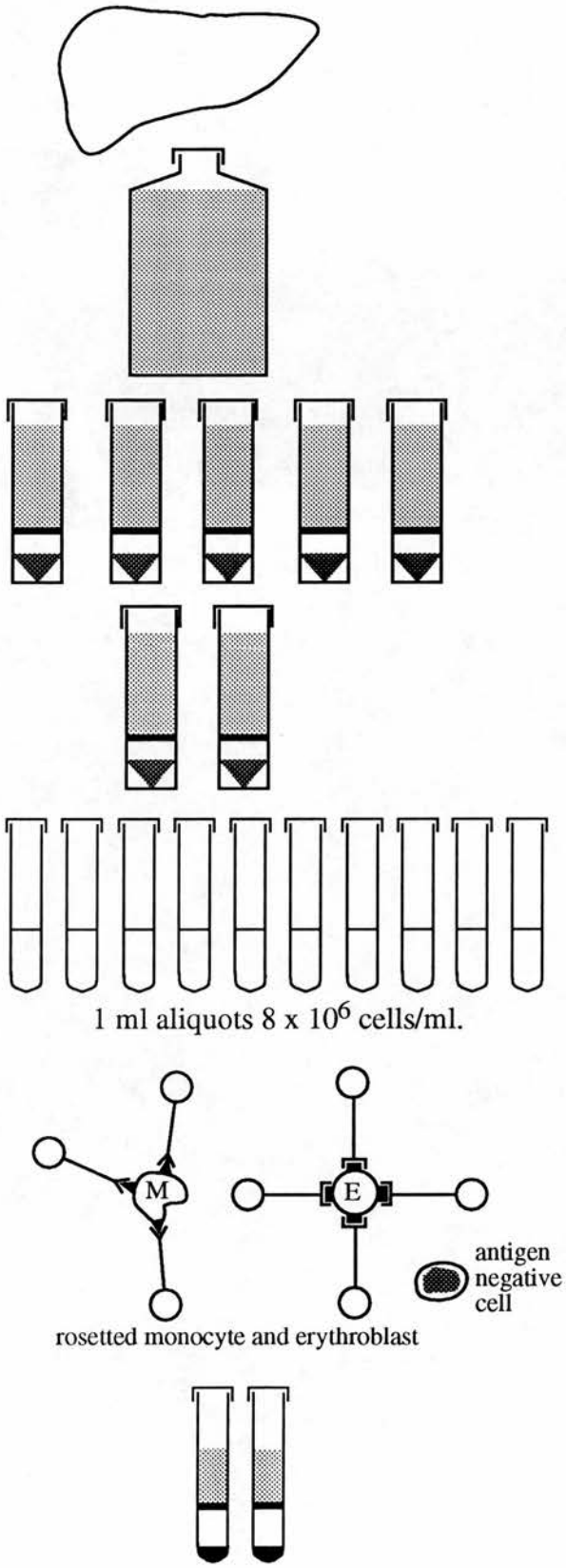


Figure 12: Schematic representation of fractionation of undifferentiated myeloid blast cells from human fetal liver following enzyme digestion of liver tissue and indirect erythrocyte rosette depletion of unwanted cells.

7.3 Erythrocyte rosette depletion of unwanted cells

The isolation of undifferentiated myeloid blast cells from human fetal liver was undertaken by the process of removing unwanted cells using the technique of indirect erythrocyte rosette sedimentation. This method is illustrated schematically in figure 12. For example, to remove erythroblasts, which express the glycophorin C antigen on their cell surface, cells were incubated with a mouse monoclonal antibody to glycophorin C (Ret40F). After washing away the primary antibody, the cells were then incubated with sheep erythrocytes coated with antibody to mouse immunoglobulin. The resulting rosetted cells were removed by centrifugation over ficoll hypaque. Antigen negative cells remaining at the interface were harvested for subsequent use.

7.3.1. Reagents used in erythrocyte depletion experiments

a) Sheep erythrocytes

Sheep erythrocytes were supplied weekly as fresh 50% whole blood in Alsever's anticoagulant (The Binding Site, UK) and were stored at 4°C prior to use.

b) Antibody to mouse immunoglobulin

The antibody to mouse immunoglobulin, used to coat sheep erythrocytes, was a DEAE chromatography purified immunoglobulin fraction of polyclonal sheep antibody to mouse IgG (PC 271, The Binding Site, UK). Purified mouse IgG had been used as the immunising agent and the antiserum had been adsorbed with human tissue. Prior to use the antibody was dialysed thoroughly against 0.9% saline to remove phosphate anions which interfere with the chromium chloride coupling of immunoglobulin to erythrocytes. The antiserum was centrifuged at 13,000 rpm (MSE Micro Centaur centrifuge; Fisons, UK) for 30 minutes to remove high molecular weight complexes and assayed for protein content by using a commercially available assay procedure (Bradford, 1976, Biorad, UK).

c) Chromic chloride solution

Chromic chloride was used to couple immunoglobulin irreversibly to sheep erythrocytes. A stock solution of 1 mg/ml in 0.9% saline was prepared (BDH, UK) and the solution was left to age for 2 months. After this time period, the pH was adjusted to 5.0 by the dropwise addition of 1M NaOH. The working solution was a 1/10 dilution of the stock solution in 0.9% saline.

d) Coating of sheep erythrocytes with antibody to mouse immunoglobulin

Antibody to mouse immunoglobulin was coupled to sheep erythrocytes by using a modification of a previously reported method (Ling and Richardson,1981). Sheep erythrocytes were washed five times in sterile 0.9% saline by centrifugation at 1000g for 10 minutes. A 10% suspension of erythrocytes in sterile saline was prepared and 1ml transferred to a sterile tube. The erythrocytes were sedimented by centrifugation at 400g for 10 minutes and the supernatant was discarded. To the red cell pellet was added that volume of the antibody to mouse immunoglobulin known to contain 0.3 mg protein. Using a vortex mixer 0.8 ml of the working chromium chloride solution (at 0.1 mg/ml in 0.9% saline) was added slowly to the erythrocyte/immunoglobulin mixture and the sides of the tube were then carefully washed down by the addition of further sterile 0.9% saline. The tube was then left overnight at 4°C. The following day the supernatant was carefully removed and discarded and the immunoglobulin coated erythrocytes were washed twice in RPMI 1640 by centrifugation at 400g for 10 minutes. The erythrocytes were finally resuspended in 4 ml of RPMI 1640 containing 100 U/ml pencillin - 100μg/ml streptomycin and 2% V/V FCS. The coated cells were stored at 4°C and were used for up to 2 months without loss of activity. Before use each batch of antibody coated erythrocytes was subjected to quality control as follows. The binding capacity of various batches of antibody coated erythrocytes against antibody coated cells was assessed using blood mononuclear cells and U937 cells which had been coated with 2 fold dilutions of a constant batch of the monoclonal

antibody BK19.45. This antibody recognises the CD45 antigen (Brown et al, 1981) which is well expressed on blood mononuclear cells and expressed to a lesser extent on U937 cells.

e) Primary antibody reagents used in rosette depletion experiments

Two mouse monoclonal antibodies were used as primary antibody reagents in the rosette depletion experiments described in this thesis. These were 1) Ret40F, a tissue culture supernatant antibody to glycophorin C which is an antigen expressed on cells of the erythroid lineage (Gatter et al, 1988). This reagent was kindly supplied by Dr D Y Mason, Oxford; and 2) 61D3, an antibody directed against an antigen expressed on monocytes and macrophages (Nunez et al, 1982). This was available as either an ascitic fluid or tissue culture supernatant from our own laboratory. All reagents contained saturating amounts of antibody as revealed by titration experiments using both the indirect rosette and indirect immunofluorescence techniques. The antibody to glycophorin C (Ret40F) was titrated against the early erythroid cell line K562 and 61D3 was titrated against peripheral blood mononuclear cells. Additional experiments adding in other primary antibodies to the depletion cocktail were undertaken but these are not described in this thesis.

f) Acridine orange solution

A saturated solution of acridine orange (Sigma, UK) was prepared by dissolving 0.1g in phosphate buffered saline, pH 7.1 and stored in the dark at 4°C. Seventy five μl of the stock solution were added to 20 mls of phosphate buffered saline, pH 7.1 to provide the working solution.

7.3.2 Erythrocyte rosette depletion technique

Following ficoll hypaque fractionation, fetal liver mononuclear cells were resuspended in RPMI 1640 containing 2% V/V FCS and saturating amounts of a mixture of selected monoclonal antibodies reactive against unwanted cell populations. Initial experiments were carried out in 500 μl aliquots at a cell density of 4 x 10^6/ml and the cells were incubated with antibodies for 15 minutes at room temperature. In later experiments the volume was increased to 1ml of cells at 8 x 10^6/ml and the incubation period was extended to 30 minutes. Following two washes in RPMI 1640 containing 2% V/V FCS, the cells were resuspended in 250µl of sheep erythrocytes coated with antibody to mouse immunoglobulin and centrifuged at 300g for 3 minutes. After a further 5 minutes incubation at 4°C, the cells were resuspended and pooled. An aliquot of 50µl was mixed with 50µl of acridine orange solution and rosette positive cells were enumerated by fluorescence microscopy using a Zeiss microscope equipped with an HB50 mercury burner for incident illumination (Ling and Richardson, 1981). Viable cells forming rosettes with ≥3 erythrocytes were classified as positive cells. The remaining cells, in 6 ml aliquots, were fractionated over 3 mls ficoll hypaque to remove rosetted cells and excess sheep erythrocytes. The remaining interface cells were harvested, washed three times in RPMI 1640 containing 2% FCS and enumerated prior to further use.

7.4 Elutriation of fetal liver mononuclear cells

Elutriation of fetal liver mononuclear cells was performed by using the JE-6B elutriation system and the standard chamber (Beckman Instruments, UK). Details describing the assembly, operation and sterilisation of the system are contained in the manufacturer's manual and the system is illustrated in figure 13. Counter current elutriation uses liquid flow versus centrifugal force to separate a suspension of single cells or particles of different size and density. A cell suspension is loaded at a constant flow rate onto the base of a chamber in a centrifuge rotor. When centrifugal and counterflow forces are balanced particles of a specific size remain at the base of the rotor chamber. Larger particles also remain in the rotor but smaller, lower density

particles and liquid are washed out through the top of the chamber. If the rotor speed is kept constant, increasing the flow rate of fluid loaded into the base of the rotor chamber will wash out increasingly larger, more dense particles. This can also be achieved by maintaining a constant flow rate and reducing the rotor speed. Cells remaining in the rotor can be collected washing out the remaining cells, the rotor off fraction. The relationship between flow rate (F), particle diameter (D) and rotor speed (R) to wash out particles of a given size is given by the formula

$$D^2 = \frac{F.X}{R^2}$$

Where X = a constant which takes into account the cross sectional area of the chamber at the elutriation boundary, the radius at the elutriation boundary, the viscosity of the suspending medium, the density difference between the particles and the suspending medium and which assumes that particles are of an equal density.

Cells suspended in RPMI 1640 containing 2% V/V FCS were loaded onto the elutriator in a volume of 4-5 ml at an initial flow rate of 4.7 ml/minute and constant rotor speed of 1950 rpm. Sterile phosphate buffered saline, pH 7.1 (Sigma, UK) containing 2% V/V FCS was used as the elutriation buffer. The flow rate was increased in increments of approximately 4.3 ml/minute up to 34.5 ml/minute and 100 ml fractions were collected at each flow rate. The rotor off fraction was also collected in some experiments. Elutriation fractions were centrifuged at 250g for 10 minutes and resuspended in RPMI 1640 containing 2% V/V FCS. Initial assessments included enumeration, cytochemical staining of cytocentrifuged preparations and marker analysis. For subsequent culture studies individual or pooled fractions were recentrifuged and resuspended at a cell density of 5×10^5/ml in the appropriate culture medium.

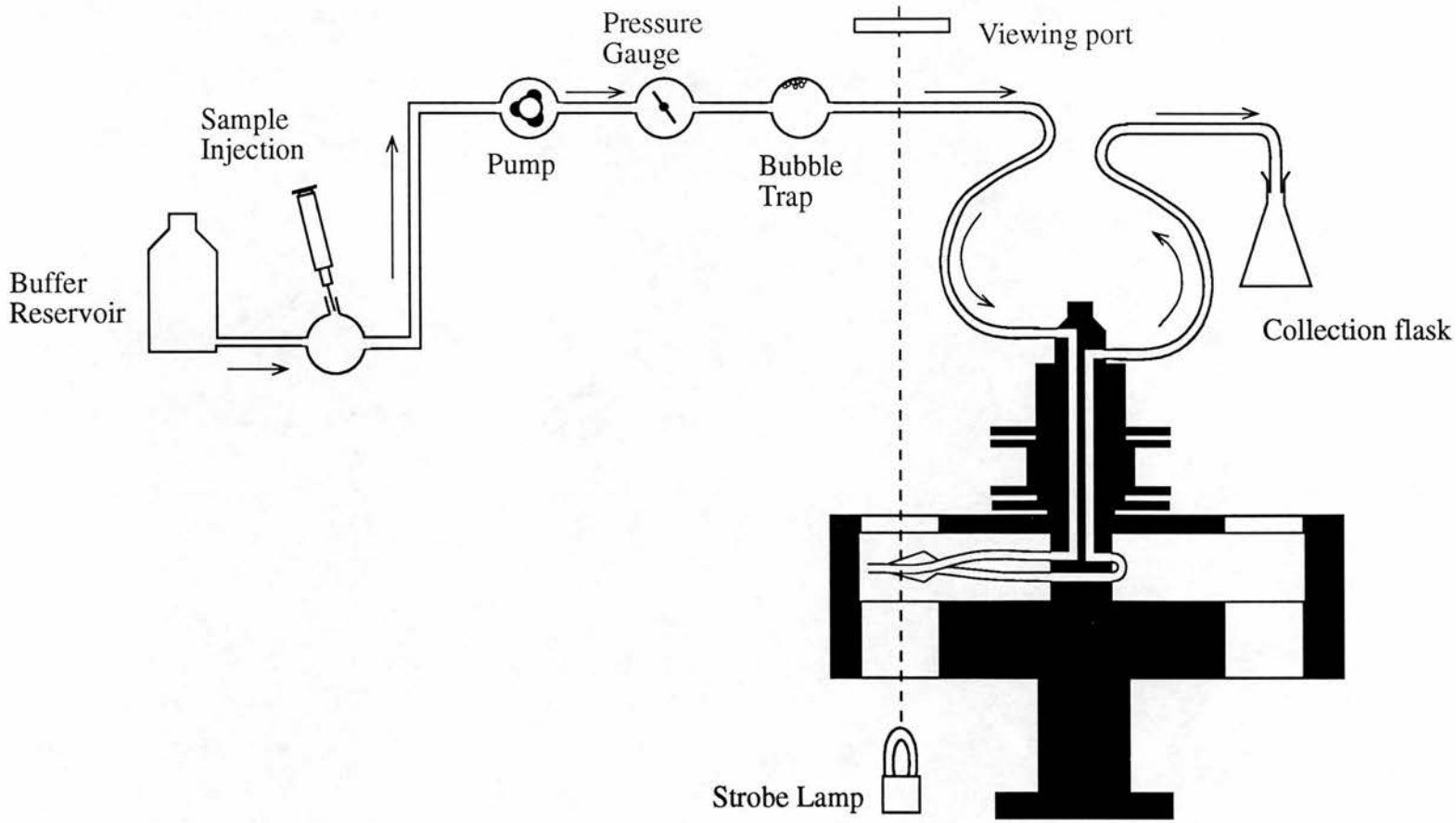


Figure 13: Schematic illustration of the Beckman JE – 6B Elutriation system. A cell suspension was loaded at a constant flow rate onto the base of the elutriation chamber where centrifugal force prevented cells from flowing to the apex of the chamber. The rotor speed was kept constant but increasing the flow rate permitted cells to flow to the top of the chamber where they were collected in fractions. Smaller, less dense cells were collected at lower flow rates but at progressively higher flow rates larger more dense cells could be collected.

CHAPTER 8.
CHARACTERISATION OF CELL POPULATIONS

8.1 Cytocentrifuge preparation of cells

Washed cells were resuspended in RPMI 1640 containing 2% V/V FCS at concentrations of 3-8 x 10^5/ml. Fifty µl aliquots of the cell suspension were cytocentrifuged for 3 minutes at 500 rpm onto clean washed slides (Cytospin II Cytocentrifuge, Shandon, UK). The slides were dried immediately and used for subsequent histochemical and immunostaining procedures. On occasions when immunostaining procedures were performed later slides were air dried for 48 hours and stored unfixed wrapped in aluminium foil at -20°C. Before use frozen slides were equilibrated with room temperature for 2 hours prior to removal of the aluminium foil.

8.2 Histochemical staining techniques

8.2.1 May-Grunwald-Giemsa

Romanowsky staining was performed by the May-Grunwald-Giemsa method (Dacie and Lewis, 1984) using an automated staining instrument (Hemastainer, Geometric Data, USA). Differential cell counts were performed on a minimum of 400 cells. In the case of the histochemical stains described below a minimum of 200 cells were also enumerated.

8.2.2. Sudan Black B

Sudan Black B staining was used to identify cells showing evidence of granulocytic or monocytic maturation. Air dried films or cytocentrifuged preparations were fixed in phosphate buffered acetone - formaldehyde for 90 seconds and stained as previously described (Dacie and Lewis, 1984). Films were then counterstained using Mayer's haemalum for 5 minutes, washed under running tap water for 5 minutes, air dried and mounted under an aqueous mountant. All histochemical staining procedures included slides containing positive control cells.

8.2.3 Chloroacetate esterase

Chloroacetate esterase staining was used to identify cells showing evidence of granulocytic maturation. Films were fixed in phosphate buffered acetone - formaldehyde as described above and washed for 10 minutes in running tap water. Three mg naphthol AS-D chloroacetate substrate (Sigma, UK) was dissolved in 1ml dimethyl formamide (Sigma, UK) in a glass container. Fifty μl of fresh 4% sodium nitrite (BDH, UK) was mixed with 50 μl of new fuchsin (Gurr, UK) and after 60 seconds 19 ml of 0.066M phosphate buffer, pH 7.6 was added to the dye mixture. The substrate and dye solutions were then mixed and the films incubated in the reaction mixture for 15 minutes at room temperature. Films were then washed under running water for 10 minutes and counterstained using Mayer's haemalum. Positive cells showed a red reaction product.

8.2.4. Monocyte specific esterase

Staining for monocyte specific esterase was carried out by two methods using either alpha naphthyl acetate or alpha naphthyl butyrate as the substrate. The diffuse positive dark red reaction pattern typical of monocytes was used to identify cells showing evidence of monocytic maturation. In contrast, a dot like reaction pattern is seen in subpopulations of T lymphocytes. The method using alpha naphthyl acetate as the substrate was performed as previously described (Yam et al, 1971). The method using alpha naphthyl butyrate is described below. Films were fixed in phosphate buffered acetone-formaldehyde as described above. Ten mg of alpha naphthyl butyrate (Sigma, UK) were dissolved in 0.5 ml 2-methoxyethanol (BDH, UK) in a glass container and added to 9.8 ml 0.067M phosphate buffer, pH 6.3. Twenty five μl of hexazotised pararosanilin HCl (Taab Laboratories, UK) were mixed with 25 μl of fresh 4% sodium nitrite and after 60 seconds mixed with the substrate solution. The slides were then incubated in the reagent mixture for 45 minutes in a 37°C water bath. Following washing with water, the slides were counterstained with either Mayer's haemalum or methyl green.

8.2.5 Periodic acid - Schiff reaction

The periodic acid - Schiff (PAS) reaction depends on the liberation of carbohydrate radicals from glycoproteins and their oxidation to aldehydes by the Schiff reagent. A positive reaction, which is not lineage specific is seen in haemopoietic cells showing signs of maturation and the strongest reactions are seen in mature neutrophilic granulocytes. Undifferentiated cells particularly myeloblasts are either negative or stain diffusely pale pink. Films were fixed in phosphate buffered acetone-formaldehyde and stained without prior diastase digestion using the method of Dacie and Lewis, 1984.

8.3 Immunophenotypic analysis of cells

A variety of immunostaining methods was used in the characterisation of cells depending on factors such as 1) the number of cells available for analysis; 2) antigen localisation and sensitivity to fixation and 3) the time available for immunostaining procedures. In all immunostaining procedures, positive, negative and substitution controls were included and a minimum of 200 cells were enumerated. A list of the antibodies used is shown in Table 9.

8.3.1 Indirect immunofluorescence technique

An indirect immunofluorescence technique was used on cytocentrifuge preparations to identify cells reacting with the monoclonal antibody AGF 4.48, which recognises neutrophilic granulocytes from promyelocyte to neutrophil stages of maturation (Fisher et al, 1982). Unfixed cytospin preparations were incubated with AGF4.48 tissue culture supernatant for 30 minutes at room temperature in a moist chamber. The slides were washed using a wash bottle and by dipping briefly into three consecutive baths of BBSS. The cells were then incubated in 50 µl of fluorescein conjugated sheep antibody to mouse immunoglobulin (own laboratory) diluted 1/50 in BBSS and containing 10% v/v normal sheep serum and 5% v/v FCS. The slides were rinsed and washed in BBSS containing 0.1% bovine serum albumin (Sigma, UK) by stirring continuously for 45 minutes. The slides were mounted in 50% glycerol

TABLE 9. Antibody reagents used in the immunophenotypic analysis of cells.

Cell Type	Antibody (Class)	Antigen Specificity	Source	Reference
Common leucocyte	BK 19.45	CD45	Own laboratory	Brown et al, 1981
Erythroid	Ret 40F (IgG_1)	Glycophorin C	Mason, Oxford	Gatter et al, 1988
Granulocyte	a) AGF 4.48 (IgM)	CD15	Own laboratory	Fisher et al, 1982
	b) PC 062 (sheep polyclonal)	Lactoferrin	Binding Site, UK	Pryzwansky et al, 1979
Monocyte	a) 61D3 (IgG_1)	unknown	Own laboratory	Nunez et al, 1982
	b) Cris6 (IgG_1)	CD14	Vilella, Madrid	Hogg et al, 1987
Granulocyte/monocyte	BU15 (IgG_1)	CD11c	Ling, Birmingham	Hogg et al,1987
T cell	a) OKT3 (IgG_2a)	CD3	ATCC[1]	Reinherz et al, 1980
	b) T3-4B5 (IgG_1)	CD3	Dako, UK	Dako, UK
	c) OKT1 (IgG_1)	CD5	ATCC	Reinherz et al, 1979
	d) 3A1 (IgG_1)	CD7	ATCC	Bernard et al, 1984
B cell	a) AL2 (rat IgG_2b)	CD10	Lebacq, Brussells	Lebacq et al, 1984
	b) 8EB1 (IgG_1)	CD19	Ling, Birmingham	Ling et al, 1987
	c) 4KB128 (IgG_1)	CD22	Dako, UK	Dako, UK
Stem cells/progenitors	a) ICH3 (IgG_2a)	CD34	Levinsky, London	Civin et al, 1989
	b) A21CB1 (IgG_1)	Class II HLA	Ling, Birmingham	Lowe et al, 1986

1 ATCC = American Type Culture Collection.

containing 25 g/l of 1,4 diazobicyclo (2.2.2) octane (Aldrich Chemical Co., UK) buffered at pH 8.6 in order to retard quenchng of the fluorescein labelled antibody when observed by fluorescence microscopy. Indirect immunofluorescence was used to identify the presence of cell surface antigens on unfixed cells in cell suspension. Fresh cells were washed three times in BBSS containing 2% v/v FCS and resuspended at 2 x 10^7cells/ml. Fifty μl of the cell suspension were incubated with 50 μl of the primary mouse monoclonal antibody reagent for 30 minutes at room temperature. The cells were washed twice in buffer and incubated with fluorescein conjugated sheep antibody to mouse immunoglobulin (as above) for 30 minutes at 4°C. After two washes in buffer the cells were resuspended in a small volume, mounted, sealed under molten wax and examined by fluorescent microscopy. For assessment of CD34 positive cells within rosette depleted fetal liver mononuclear cells an FITC conjugated antibody to mouse IgG2a was used to avoid staining of residual monoclonal antibodies. The antibodies used in depletion experiments were of isotypes other than IgG2a.

8.3.2. Indirect immunoperoxidase technique

An indirect immunoperoxidase technique on cytocentrifuged preparations was used to stain for the presence of lactoferrin, a known constituent of neutrophil secondary granules (Pryzwansky et al, 1979). Fixation and blocking of endogenous peroxidase activity was achieved by incubation for 20 minutes in methanol formalin (1:1) containing 0.6% 30% v/v hydrogen peroxide (BDH, UK). After washing, incubation for 20 minutes at room temperature with a mixture of 20% normal human AB serum and 20% swine serum was used to prevent non specific binding. The preparations were then incubated for 30 minutes at room temperature with a 1:50 dilution of an IgG fraction of a polyclonal sheep antibody to lactoferrin (PCO62), The Binding Site, UK). After washing, the slides were incubated for a further 30 minutes with a 1/100 dilution of a horse radish peroxidase conjugated swine antibody to sheep immunoglobulin (PP361, The Binding Site, UK). After washing and colour development with 3,3 diaminobenzidine, nuclei were counterstained with Mayer's haemalum. Slides were mounted in DPX mountant (BDH, UK) and

examined under light microscopy. Phosphate buffered saline, pH 7.1 containing 0.1% BSA was the dilution and washing buffer used in the above staining procedure.

8.3.3. Immuno alkaline phosphatase technique

Immunocytochemical staining for mature T and B lymphocytes was performed on cytocentrifuge preparations by the immunoalkaline phosphatase technique using monoclonal antibodies to the CD3 and CD22 antigens as previously described (Erber et al, 1984). To prevent non specific binding, 0.1% BSA was added to the wash and dilution buffers and dilutions of the primary and secondary antibody were supplemented with 10% normal rabbit serum. The CD3 antibody (M756, diluted 1 in 100), CD22 antibody (M738, diluted 1 in 200), rabbit antibody to mouse immunoglobulin (Z259, diluted 1 in 25) and alkaline phosphatase : anti-alkaline phosphatase (APAAP) complexes (D651, diluted 1 in 50) were purchased from Dako, UK. To enhance the sensitivity a 10 minute repeat incubation step with both the antibody to mouse immunoglobulin and the APAAP complexes was performed.

8.3.4. Direct erythrocyte rosette technique

Cell surface antigens may be detected by a simple direct technique using sheep erythrocytes coupled with a primary antibody (Ling and Richardson, 1981). This is a routine method employed in the immuno-diagnostic laboratory, Department of Immunology, University of Birmingham and sheep erythrocytes coupled with primary antibodies were kindly provided by Mr P R Richardson. These reagents were daily checked against positive and negative control cells. The assay was performed to determine directly the number of cells expressing a particular antigen when cells had been previously coated with monoclonal antibodies for cell depletion experiments. In this case indirect assays give a potentially inaccurate measure of the number of positive cells due to the presence of residual unseparated antibody coated cells. Thirty μl of a 5% suspension of primary antibody coated cells were added to 100 μl of test cells suspended in RPMI 1640 containing 2% FCS at 6 x 10^5/ml. The cells were centrifuged at 250g

for 3 minutes and incubated for a further 5 minutes at 4°C. Fifty μl of acridine orange solution was then added and rosette positive cells enumerated as previously described.

8.3.5. Indirect erythrocyte rosette technique

The principle and practice of the indirect erythrocyte rosette technique is identical to that used in the rosette depletion of unwanted cells from human fetal liver. For small scale assays, a saturating amount of primary antibody was added to 50 μl of a cell suspension at 2 x 10^6/ml. The cells were incubated for 15 minutes at room temperature with gentle agitation and washed as previously described. Thirty μl of sheep erythrocytes coated with antibody to mouse immunoglobulin were then added and the enumeration of rosette positive cells was carried out as previously described.

8.4 Physiological assays

8.4.1 Analysis of the phagocytic capacity of cells

The ability of cells to phagocytose complement-coated yeast cells was performed as previously described (Toksoz et al, 1982). Glutaraldehyde fixed yeasts (Saccharomyces cerevisiae) were mixed in 0.2% procion rubine in 0.2M Na_2HPO_4 for 2 hours at room temperature. The dyed yeasts were then washed free of surplus dye in complement fixation test buffer (CFT, Oxoid, UK). For complement coating 2 x 10^8 organisms were suspended in 800 μl of CFT and 200 μl of fresh normal sheep serum was added. The mixture was incubated for 30 minutes at 37°C and agitated at 5 minute intervals to keep the yeasts suspended. The yeasts were then washed twice and resuspended in 20 mls CFT and stored as 0.5 ml aliquots at -20°C. For the phagocytosis assay 5 x 10^4 cells were washed in RPMI 1640 containing 10% v/v FCS and the supernatant discarded. To the pelleted cells were added 50 μl of RPMI 1640 containing 10% v/v FCS and 10% v/v fresh human AB serum and 50 μl of the opsonised yeast suspension. The mixture was incubated at 37°C for 30 minutes and then mounted in a haemocytometer. Viable

mononuclear cells seen to contain ≥ 3 intracellular dyed yeasts were enumerated as phagocytic cells.

8.4.2. Analysis of the colony forming ability of cells

The number of cells able to form colonies in semi-solid medium was determined using a method similar to that described by Ash et al, 1981. In the case of a colony forming unit - granulocyte macrophage (CFU-GM) assay 2×10^4 - 2×10^5 mononuclear cells were seeded in duplicate in a 1.0 ml volume of IMDM containing 1.2% methyl cellulose (Sigma, UK), 20% v/v FCS, 1% de-ionised bovine serum albumin, 5×10^{-5}M 2-mercaptoethanol (Sigma, UK), 5% phyto-haemagglutinin stimulated leucocyte conditioned medium (PHA-LCM) and 100 U/ml penicillin - 100 μg/ml streptomycin. Colonies containing ≥40 cells were enumerated at day 12. For assessment of blast forming unit-erythroid (BFU-E) colonies an identical system containing, in addition, erythropoietin (Eprex, Cilag, UK) at 3 U/ml was used and the cells were plated at densities ranging from 0.2 - 2×10^5/ml. Clusters of ≥3 erythroid colonies or single erythroid colonies containing >300 cells were enumerated as BFU-E. The nature of sample colonies was confirmed by staining cytocentrifuged preparations of single colony contents harvested by micropipette under a dissecting microscope.

The phytohaemagglutinin stimulated leucocyte conditioned medium (PHA-LCM) had been prepared as follows. A fresh donation of whole blood collected into citrate phosphate dextrose adenine from a normal donor was fractionated by using ficoll hypaque on an IBM 2001 cell separator to generate a mononuclear cell suspension. Ten ml aliquots of cells suspended at 2×10^6/ml in IMDM containing 10% v/v FCS, 2 mM/l glutamine (Gibco,UK), 100U/ml penicillin/100μg/ml streptomycin and 1 mg phytohaemagglutinin (Wellcome, UK) were cultured in 25 cm^2 tissue culture flasks at 37°C in a humidified atmosphere containing 5% CO_2 for 5 days. The tissue culture supernatants were then harvested, pooled, sterilised by microfiltration, aliquotted in small volumes and stored at -20°C. A single unit of blood generated a batch of 50ml PHA-LCM which was used for all the colony assays. The PHA-LCM was titrated in CFU-GM and BFU-E assays of normal bone marrow

mononuclear cells and used at an optimal concentration of 5%. The bovine serum albumin, methylcellulose, fetal calf serum and 2-mercaptoethanol had also been prepared as single large batches sufficient for all assays and were stored at -20°C. The de-ionised BSA was prepared as a 10% stock solution as follows. Ten g of BSA (Sigma, UK) were dissolved in 44 ml H_2O overnight at 4°C. Deionisation was performed using an analytical grade mixed bed resin type AG501 - X8(D) (Biorad, UK) and the resulting solution was freeze dried overnight. The albumin was then dissolved in 94 ml IMDM containing 100 U/ml penicillin and 100 μg/ml streptomycin, filter sterilised, aliquotted in 1ml volumes and stored at - 20°C.

8.4.3. Analysis of the phase of the cell cycle

Mononuclear cells fractionated from human fetal liver and rosette depleted using the Ret40F and 61D3 antibodies were assayed for the percentages of cells in G_0/G_1, S, and G_2/M phases of the cell cycle. The assay was based on the principle that flow cytometric analysis of propidium iodide stained nuclei represents DNA content. Test cells were prepared at 2 x 10^6/ml, processed using a commercial kit (Cycletest, Becton Dickinson, UK) and analysed using commercial software (Cellfit, Becton Dickinson, UK) in a flow cytometer (FACScan, Becton Dickinson, UK). For comparison analyses were simultaneously performed on HL60 cells and G_0 lymphocytes isolated from human tonsils.

CHAPTER 9.
STUDIES USING BLAST CELLS PURIFIED FROM HUMAN FETAL LIVER.

9.1.1. Culture of fetal liver cells in medium containing fetal calf serum.

Blast cells purified from fetal liver following mechanical disruption of tissue and rosette depletion using Ret40F and 61D3 monoclonal antibodies (directed against glycophorin C and a macrophage associated antigen) were cultured in RPMI 1640 medium containing 20% v/v heat inactivated FCS, 10μM hydrocortisone sodium succinate (Upjohn, UK) 5 μg/ml vitamin D_3 (cholecalciferol, Sigma UK) and 100 U/ml penicillin - 100 μg/ml streptomycin. Cells were seeded at a density of 2.5 x 10^5/ml in 1ml volumes in individual wells of 24 well tissue culture plates (Nunc, UK). This medium had been previously shown to support the growth and differentiation in liquid suspension culture of human fetal liver cells isolated following mechanical disruption and one step ficoll hypaque fractionation (Toksoz and Brown, 1984).

9.1.2 Culture of fetal liver cells under serum-free conditions

Fetal liver blast cells were cultured under serum free conditions with and without added IL-3 to a) promote the survival and proliferation of undifferentiated blast cells; b) permit biochemical studies of inositol metabolism in cells grown in medium containing inositol at 1 mg/l and [^{3}H]-inositol and c) to eliminate the interference of differentiation factors contained within fetal calf serum in the biochemical studies. Fetal liver cells purified by enzyme digestion, ficoll fractionation, Ret40F and 61D3 rosette depletion and elutriation were used for these studies. Cells were cultured in RPMI 1640 medium containing 100 U/ml penicillin - 100 μg/ml streptomycin and with and without 100 U/ml human recombinant interleukin 3 (IL-3, Genzyme, USA) in 1ml volumes, at 5 x 10^5/ml in individual wells of 24 well tissue culture plates.

9.1.3 Induction of differentiation by phorbol myristate acetate

Phorbol myristate acetate (PMA) is known to induce rapid monocyte maturation of the human promyeloid cell line, HL60 (Rovera et al, 1979).

The effect of PMA on the differentiation of normal blast cells purified from human fetal liver was studied. Cultures were established in 24 well tissue culture plates as described above. PMA treated wells contained 10 nM PMA (Sigma, UK).

9.2 Assessment of fetal liver cultures

The fetal liver cultures described above were characterised using the procedures outlined below. Individual wells were harvested at various time points ranging from 12 hours to 7 days. During this procedure wells were rinsed with cold tissue culture medium to ensure that all cells had been harvested. This was particularly important in the case of PMA treated cultures where significant numbers of adherent cells were present. The contents from harvested wells were centrifuged and resuspended in their initial volume prior to assessment of cell count and viability. Cell viability was assessed by phase contrast microscopy.

9.2.1. Assessment of cell growth

The effect of PMA treatment on the proliferation of blast cells purified from fetal liver was determined by measuring DNA synthesis by the incorporation of [^{3}H]-thymidine. These studies were undertaken on blast cells purified following mechanical disruption of tissue and rosette depletion using the Ret40F and 61D3 monoclonal antibodies. Cells were seeded at 1 x 10^5 per well in 100 µl volumes of medium containing fetal calf serum in flat bottomed wells of 96 well microtitre plates (Nunc, UK). PMA treated wells contained 10 nM PMA. 0.5 µCi of [^{3}H]-thymidine (25 Ci/mM; Amersham International, UK) was added in 50 µl of medium at 0, 12, 36, 60 and 84 hours and cultures were harvested 6 hours later on a Skatron automatic harvester (Flow Laboratories, UK). [^{3}H]-thymidine incorporation was measured by scintillation counting and incorporation determinations were performed in triplicate.

9.2.2. Assessment of cell differentiation

The degree of differentiation of cultures was assessed using a combination of May-Grunwald-Giemsa morphology, histochemical

staining methods and immunophenotypic analysis as described in Chapter 8. In particular, granulocyte differentiation was assessed by 1) the percentage of cells at the promyelocyte to neutrophil stage of maturation following staining with MGG; 2) the percentage of cells staining with the AGF4.48 antibody and 3) the percentage of cells staining with antibody to lactoferrin. Monocyte maturation was assessed by 1) the percentage of cells at the promonocyte to monocyte stage of maturation following staining with MGG and 2) the percentage of cells showing positive staining for monocyte specific esterase.

9.3. Analysis of intracellular inositol phosphates

These studies were undertaken in collaboration with Dr C Bunce and Dr P French, Department of Biochemistry, University of Birmingham. Previous studies by Dr C Bunce and Dr P French on HL60 cells showed that these cells could be labelled to equilibrium with [^{3}H]-inositol within 4-5 days of culture following which the concentrations of intracellular inositol phosphates could be measured. Further more, following equilibrium labelling with [^{3}H]-inositol, HL60 cell monocyte differentiation could be induced by PMA and the changes in intracellular inositol phosphate concentrations observed. For studies of inositol metabolism on blast cells purified from human fetal liver, cells were cultured in inositol free RPMI 1640 medium (Northumbria Biologicals, UK) supplemented with 1 mg/l inositol (Gibco, UK), 2 μCi/ml [^{3}H] myo-inositol (Amersham International, UK), 2mM/l glutamine (Gibco, UK), 100 U/ml IL-3 and 100 U/ml penicillin 100 μg/ml streptomycin. The [^{3}H]-inositol was supplied in a 90% ethanol/10% water diluent. As the diluent had a detrimental effect on cells, the label was freeze dried, dissolved in tissue culture medium and filter sterilised prior to use. Cells were cultured at 5 x10^5/ml in 1ml volumes in 24 well tissue culture plates. On day 2, the cultures were fed by removing 0.5 ml of supernatant medium and replacing this with 0.5ml fresh medium and after 5 days, to permit labelling to equilibrium, individual wells were harvested for analysis of intracellular phosphates. To study the effect of PMA on intracellular inositol phosphate concentrations, PMA (10 nM) was added after 5 days culture and the individual wells harvested after culture for a further 24 hours.

Intracellular [^{3}H]-inositol phosphates were analysed following a process of acid extraction and separation using anion exchange high performance liquid chromatography (HPLC). A cell suspension of known volume and cell counts was centrifuged at 200 g for 10 minutes and washed once in inositol-free RPMI 1640. 0.5ml of 20% w/v trichloroacetic acid (containing 250 μg/ml phytic acid and the equivalent of 4 μg phosphate/ml of a phytic acid hydrolysate; Wreggett and Irvine, 1987) was added to the cell pellet. Following mixing, the contents were transferred to a polypropylene vial and the original centrifuge tube was washed again with 0.3 ml 2% w/v trichloroacetic acid (TCA). The two solutions were combined and centrifuged at 3000 g for 5 minutes. The supernatant was retained and the precipitate washed with 0.2 ml of 2%w/v TCA. The two supernatants were combined and the TCA neutralised by extracting it into methyl ether (4 washes with vigorous mixing). The aqueous layer was retained and 100 μl of 50 mM EDTA (pH 7.0) were added to prevent precipitation of salts of inositol phosphates and the extract stored at -20°C. [^{3}H]-inositol phosphates were separated using a LDC/Milton Roy gradient elution system on a Partisil 10SAX strong anion exchange column. Inositol phosphates are negatively charged and bind to the positively charged column. The column was eluted with increasing concentrations of ammonium phosphate which displaced increasingly higher inositol polyphosphates. The Partisil 10SAX column (25 cm x 4.6 mm) was used with a pre-column (Whatman 37-53 microns pre-column gel, 5 cm x 4.6 mm) and a guard column (Whatman Pellucular Anion exchanger, 5 cm x 4.6 mm). The following gradient was employed using water in pump A and initially 0.1M ammonium phosphate (pH = 3.8) in pump B changing over to 1M ammonium phosphate (pH = 3.8) in pump B at 65 minutes.

Time (minutes)	% B
0	0
2	0
35	30
35.1	100
65	100
65.1	30
95	30

Time (minutes)	% B
95.1	70
110	70
110.1	80
120	80
120.1	100
145	100
145.1	0

The column was re-equilibrated with water for at least 30 minutes prior to re-use. The flow rate was 1ml/minute and fractions were taken every 0.5 minutes. The radioactivity of each fraction was measured using a Packard liquid scintillation counter (model No.2000CA), following the addition of scintillant to each vial (0.6% PPO, 0.015% POPOP in xylene mixed 1:1 with triton X 100). From knowledge of the specific activity of [^{3}H]-inositol in the medium, the number of cells analysed, the total amount of each inositol phosphate extracted and measurement of individual cell diameters an estimation of the concentration of each inositol polyphosphate per cell was made. A mean cell volume was calculated following measurements of diameters of 100 cells and the assumption that the cells were spheres. Individual cell diameters were measured under a light microscope containing a graticule which had been pre-calibrated.

CHAPTER 10.

STUDIES USING CELLS FROM A PATIENT WITH PRIMARY MYELOFIBROSIS.

10.1 Initiation of haematopoiesis on irradiated stroma from long term bone marrow cultures from a normal donor.

Fresh peripheral blood mononuclear cells (PBMNC) from the patient with primary myelofibrosis were tested for their ability to initiate haematopoiesis on irradiated stroma from long term bone marrow cultures (LTBMC) grown from a normal donor. A 10 ml long term bone marrow culture containing 2 x 10^7 marrow buffy coat cells from a normal marrow donor was established and maintained as previously described (Gartner and Kaplan, 1980). The LTBMC medium consisted of IMDM containing 10% v/v FCS, 10% v/v horse serum (Flow Laboratories, UK), 5 x 10^{-7}M hydrocortisone sodium succinate (Solu-cortef, Upjohn, UK) and 100 U/ml penicillin - 100 μg/ml streptomycin. To establish the long term bone marrow cultures, 2 mls of fresh marrow were collected into 5 mls heparinised IMDM. The volume was made up to 14 mls with medium and 2 mls hespan added. Following 60 minutes incubation at 37°C the buffy coat was harvested and washed 3 times as described previously. A 10 ml culture containing 2 x 10^7 cells was established in a 25 cm^2 tissue culture flask (Nunc, UK). The cultures were fed weekly by removal of half of the supernatant medium and replacement with fresh LTBMC medium. After 30 days non adherent cells were removed, the adherent cells trypsinised, irradiated with 15 Gy (Co 60 gamma rays, mean energy 1.25 MeV, dose rate 5.3 Gy/minute) and 3 ml of cells at 1 x 10^5/ml reseeded (at 3 x $10^4/cm^2$) in each of three 35 mm diameter wells of a 6 well tissue culture plate (Nunc, UK). After 5 days incubation, during which a healthy stroma had re-established, 1.5 ml of supernatant was removed from each of the 3 wells and to each of 2 wells was added 1.5 ml of LTBMC medium containing 5.4 x 10^6 fresh PMF PBMNC. To the remaining well was added 1.5 ml LTBMC medium alone to act as a negative control. To each of two other empty 35 mm diameter wells was added 5.4 x 10^6 fresh PMF PBMNC in 3 ml of LTBMC medium to act as a PMF control. The 5 cultures were fed weekly by removing half of the supernatant and

replacing this with fresh LTBMC medium. After 5 weeks all non adherent cells were removed and the cells in the adherent layer harvested by trypsinisation. Non adherent and adherent cells were pooled, washed, counted and aliquots removed for cytospin analysis and progenitor assays as described previously. The total number of cells harvested from each culture was as follows - PMF PBMNC alone 11 x 10^5 and 14 x 10^5; co-cultures of PBMNC and irradiated stroma 15 x 10^5 and 17 x 10^5 and irradiated stroma alone 1.4 x 10^5 cells. The cells in the stroma alone culture were plated in duplicate at a density of 0.7 x 10^5/ml in an erythroid colony assay. Otherwise cells from the other cultures were plated in triplicate in separate assays for CFU-GM and BFU-E at densities ranging from 1.5 x 10^5 - 2.3 x 10^5/ml.

10.2. Establishment of primary fibroblast lines

Primary fibroblast cultures were generated from a skin biopsy from the patient with primary myelofibrosis to provide a source of constitutive DNA. The procedure was carried out with the assistance of Dr A Sanderson, Department of Pathology. The skin biopsy was placed in a sterile petri dish and cut into small fragments of approximately 1 mm^2 using a scalpel blade. These biopsy fragments were then attached onto roughened areas on the inner surface of 60 mm diameter petri dishes which had been scored using a scalpel blade. The tissue was allowed to dry for 5 minutes to permit good attachment and the fragments were covered with Hams F10 medium containing 10% v/v FCS and 100 U/ml penicillin - 100 μg/ml streptomycin. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and fed every 3 days by careful complete replacement of the medium. When the fibroblast layers were confluent the cells were harvested using trypsin as follows. The cells were washed twice with serum-free tissue culture medium and then treated with 5 mls 0.4% trypsin in RPMI 1640 containing 1mM EDTA (Gibco, UK). The cells were left for 5-10 minutes at 37°C until detachment had occurred and the trypsin action was neutralised by adding an equal volume of culture medium containing FCS. After washing, the cells were subcultured in 80 cm^2 tissue culture flasks and grown by repeated subculture to generate sufficient

cells for cytogenetic analysis and DNA isolation. Cultured fibroblasts were also cryopreserved at -70°C as described previously.

10.3 Establishment of Epstein-Barr virus (EBV) transformed B cell lines

Epstein-Barr virus transformed B cell lines were generated from the patient with primary myelofibrosis and then subjected to karyotypic analysis to determine whether any of the lines isolated carried the same karyotype abnormality as the patient's cells. The cell lines were produced in collaboration with Dr C Gregory, Department of Immunology. Peripheral blood mononuclear cells were resuspended in warm RPMI 1640 containing 10% v/v FCS (RPMI-10) and an optimal dilution of concentrated cell free supernatant from the EBV producing B95.8 marmoset cell line (Miller and Lipman, 1973). The potency of the virus present within individual batches of B95.8 supernatant was monitored using the fetal cord blood transformation assay (Moss and Pope, 1972). Cells were infected at 37°C for 1-2 hours after which time they were washed once with RPMI 10 at 37°C, resuspended in RPMI 10 and seeded into 2 ml tissue culture wells at a concentration of 2.5×10^6 cells/ml. Regression of cultures by activated EBV specific T cells was prevented by including 5μg/ml PHA in the culture medium. The wells were fed every 3 days with fresh RPMI 10. EBV transformed cell lines were established in 3 weeks at which point the cultures were transferred to 50 cm^2 tissue culture flasks, subjected to karyotypic analysis and cryopreserved as described previously.

10.4 Karyotype analysis

Karyotypic analyses were undertaken on peripheral blood mononuclear cells, pooled GM colonies grown from PBMNC, cultured skin fibroblasts and EBV transformed B cell lines isolated from the patient with primary myelofibrosis. These studies were undertaken in collaboration with Mr S Larkins, Department of Cytogenetics, Birmingham Maternity Hospital, Dr A Sanderson, Department of Pathology and Dr C Bunce, Department of Immunology.

For analysis of peripheral blood mononuclear cells unstimulated cultures were prepared by adding 0.2 ml of peripheral blood to 5 ml of RPMI 1640 containing 10% v/v FCS and P/S. After 24 hours 0.2 μg/l colcemid (Gibco, UK) was added and incubation continued for either 1 hour or 24 hours. The cells were transferred to a conical test tube, centrifuged at 150g for 10 minutes at room temperature and resuspended in 10 mls 0.075 M KCl. After 10 minutes at 37°C the cells were centrifuged as above and the KCl removed. The cell pellet was loosened and fresh fixative (3:1, methanol:glacial acetic acid) was added dropwise whilst gently mixing the cells. After 5 mls of fixative had been added in this way the column was made up to 10 mls and the cells centrifuged as above. The fixed cells were resuspended in fresh fixative and centrifuged three more times and finally resuspended in fixative to give a slightly cloudy suspension. Two drops of the cell suspension were then dropped from a Pasteur pipette from a height of about 0.5m onto microscope slides which had been cleaned in methanol and wiped dry with clean tissue paper. Fresh slides were aged by either treatment with hydrogen peroxide or baking overnight at 60°C and banded using a trypsin-Giemsa method. Briefly slides were rinsed in phosphate buffered saline, pH 7.1, covered with 2.8% trypsin for 10-40 seconds, rinsed with buffer and then stained with Giemsa for 3-4 minutes. Stained slides were washed in running tap water, dried and mounted in DPX mountant. Metaphase analysis was performed by Mr S Larkins and Dr A Sanderson according to the international system for human cytogenetics nomenclature.

To demonstrate any cytogenetic abnormality in CFU-GM isolated from the patient, pooled GM colonies grown from PBMNC were harvested into IMDM containing 20% v/v FCS and 10% v/v PHA-LCM and incubated overnight at 37°C in 5% CO_2. Colcemid 0.2 μg/l was added and after further incubation for both 1 hour and 24 hours cells were harvested and processed as described above.

For karyotypic analysis of cultured skin fibroblasts and EBV transformed cell lines, cells were incubated with 0.2 μg/l colcemid for 1 hour prior to harvesting and processing as described above.

10.5 Determination of the involvement of proto-oncogene SEA in the t(2;11)(q24/31;q13) translocation observed in the patient with primary myelofibrosis.

Karyotypic study of PBMNC from the patient with PMF revealed a complex karyotype including the chromosomal translocation t(2;11)(q24/31;q13). Evidence from this case and a previously reported case (Partenen et al, 1982) suggested a causal relationship between an abnormality on the long arm of chromosome 11 and defective erythropoiesis in PMF. The breakpoint in the case described above (11q13) was also found to be the location of the human homologue of the viral oncogene SEA of the S13 avian erythroblastosis virus. Therefore the involvement of the proto-oncogene SEA (pSEA) in this translocation was studied at the molecular level. These studies were performed by Dr Ruth Jarrett of the Leukaemia Research Fund Virus Centre of the University of Glasgow by the analysis of DNA which was obtained from patients PBMNC and skin fibroblasts. Following restriction endonuclease digestion and Southern blotting, DNA was hybridised with a ^{32}P labelled DNA probe for human pSEA. The methods are described briefly below.

High molecular weight DNA was extracted using SDS and proteinase K as previously described (Trainor et al, 1982). Ten micrograms of DNA, from either peripheral blood mononuclear cells or skin fibroblasts were digested for 15h with the restriction endonucleases BamH1, EcoRI or Sst1 using the conditions recommended by the manufacturer. The digestion products were subjected to electrophoresis overnight at 32V in 0.8% agarose and transferred to Hybond-N membrane (Amersham International, UK) by capillary transfer. Filters were subsequently hybridised for 15h at 37° in 50% formamide/0.45M NaCl with the pSEA probe which had been previously labelled to a specific activity of 1 x 10^9cpm/μg DNA using a modification of the method of Feinberg and Vogelstein, 1983. After hybridisation, filters were washed extensively with 0.5 x SSC, 0.1% SDS at 65°C (1 x SSC is 0.15 M NaCl, 0.015M sodium citrate, pH7). Hybridising bands were detected by auto-radiography at -70°C with Hyperfilm-MP (Amersham International, UK) in a cassette using a Du Pont Quanta III screen.

The pSEA probe was obtained from the UK DNA probe bank. This is a 4.5kb cDNA of the human homolog of the S13 avian erythroblastosis oncogene cloned in EcoRI site of pvc13. This DNA sequence has been mapped to chromosome 11q13 (Williams et al, 1987).

SECTION 3. RESULTS.

CHAPTER 11. PURIFICATION OF UNDIFFERENTIATED BLAST CELLS FROM HUMAN FETAL LIVER.

11.1 Purification of undifferentiated blast cells from human fetal liver following mechanical disruption of tissue.

In initial experiments a cell suspension was prepared from fetal liver following mechanical disruption of tissue (see Materials and Methods). A mononuclear cell fraction was then obtained by a single ficoll-hypaque fractionation of cells. The total yields of cells obtained following mechanical disruption of fetal tissue and subsequent one step ficoll separation are shown below.

Table 10. Yield of mononuclear cells from fetal liver following mechanical disruption of tissue.

Number of cells		
Mean[1]	(95% CI)	Range
3.8×10^7	(3.1 - 4.6)	$0.54 - 16.0 \times 10^7$

1. Number of experiments = 58.

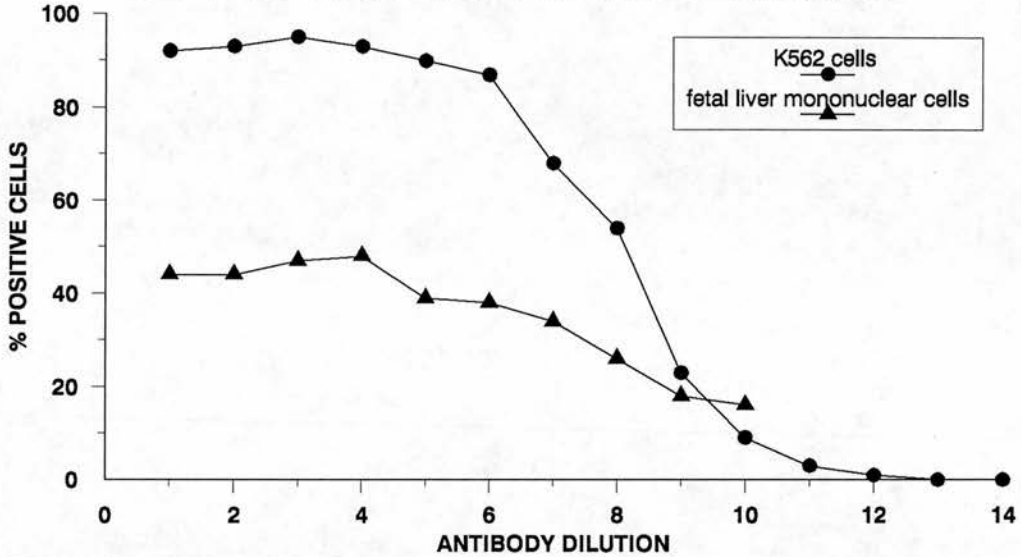
The mean of values obtained for cell yield was 3.8×10^7 cells per liver and the yield was generally related to the size of the liver. Numbers of cells obtained did not vary in relation to the time period which had elapsed between the delivery of the fetus. As to this time period, the fetuses were stored at 4°C prior to collection which ensured that the livers were in a good physical condition at the time of dissection. Assessment of the morphology of cells obtained at this stage, by staining cytocentrifuged preparations with May-Grunwald-Giemsa (MGG), is shown in Table 12 and illustrated in Plate 1a. The major populations consisted of undifferentiated blast cells (48.6%) and erythroblasts (27.4%). The remaining cells consisted of cells of the granulocytic and monocytic lineages (12.3%), cells with morphological

appearance of lymphocytes (8.9%) and cells which were not readily identified and which were classed as others (2.8%).

To obtain a population of cells which was enriched with undifferentiated blast cells, contaminating erythroblasts and monocytes/macrophages were removed by indirect rosette sedimentation, after coating cells with monoclonal antibodies directed against glycophorin C (Ret40F) and a monocyte associated antigen (61D3). To ensure that coating antibodies were used in saturating amounts in the rosette depletion experiments, antibody batches were titrated against both positive control cells and fetal liver mononuclear cells by using the indirect rosette technique. Sample titration data, obtained from tissue culture supernatants from the Ret40F and 61D3 hybridomas are shown in Figures 14 and 15. When used in rosette depletion procedures, the Ret40F and 61D3 antibodies were used at dilutions which were on the plateau portions of the titration curves.

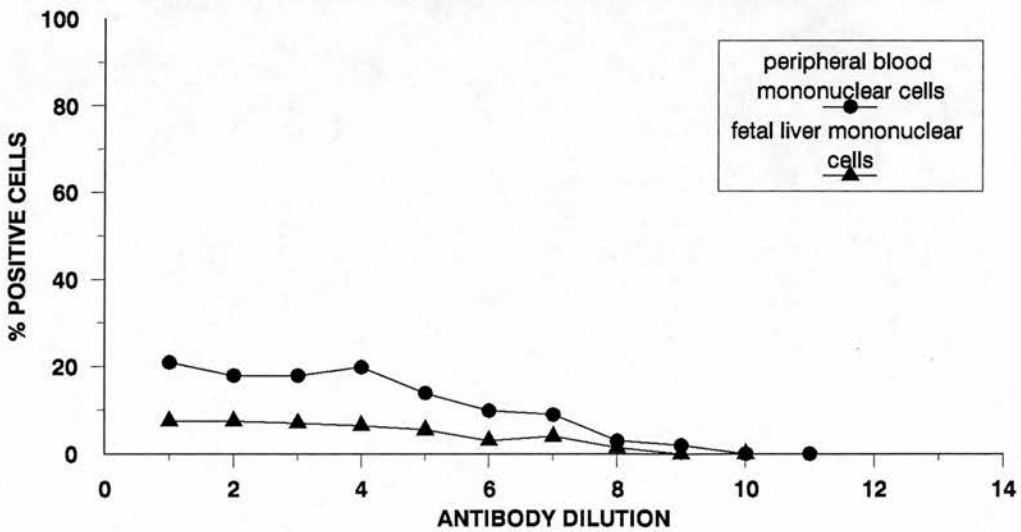
The batches of sheep erythrocytes coated with antibody to mouse IgG, which were used to rosette fetal liver mononuclear cells coated with a primary mouse monoclonal antibody were also subjected to quality control procedures. A monoclonal antibody directed against the common leucocyte antigen (BK19.45) was used as a test reagent to check batches of sheep erythrocytes. The common leucocyte antigen (CD45) is well expressed by peripheral blood mononuclear cells and weakly expressed by the promonocyte line U937. A large batch of supernatant from the BK19.45 hybridoma cell line was first titrated against peripheral blood mononuclear cells and U937 cells, by using the indirect immunofluorescence technique, to ensure that the batch of supernatant contained adequate and saturating amounts of antibody. Peripheral blood mononuclear cells were very strongly stained by BK19.45 antibody and U937 cells showed weak staining. The difference in the level of antigen expressed by the two cell types is also reflected by differences in the titre of BK19.45 antibody against U937 and peripheral blood mononuclear cells (Figures 16 and 17). The batch of supernatant was titrated at the same time against both cell types using the indirect rosetting technique and an initial batch of coated erythrocytes. As shown in Figures 16 and 17 and as revealed by the

FIGURE 14. TITRATION OF THE MONOCLONAL ANTIBODY RET40F BY THE INDIRECT ROSETTE TECHNIQUE.



Tissue culture supernatant was titrated against K562 cells and fetal liver mononuclear cells.

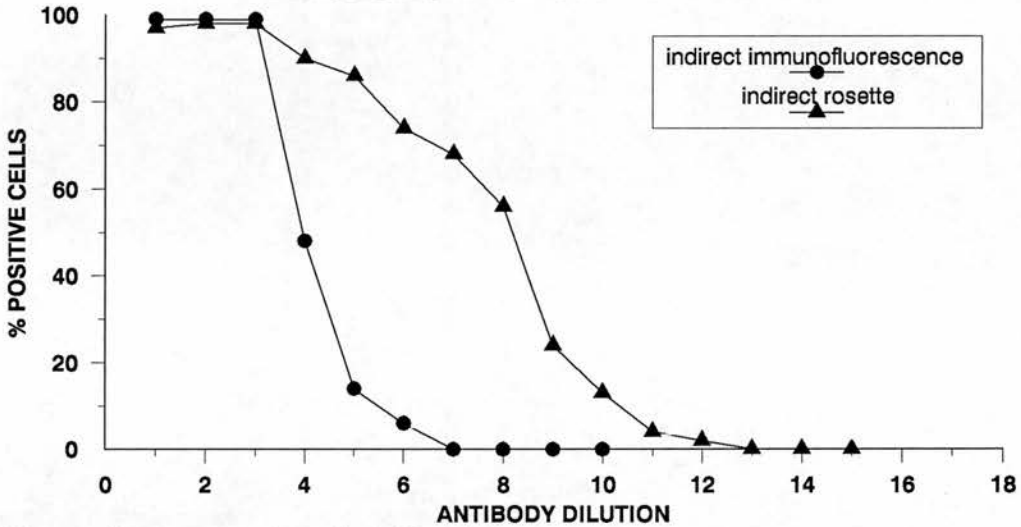
FIGURE 15. TITRATION OF THE MONOCLONAL ANTIBODY 61D3 BY THE INDIRECT ROSETTE TECHNIQUE.



Tissue culture supernatant was titrated against peripheral blood mononuclear cells and fetal liver mononuclear cells.

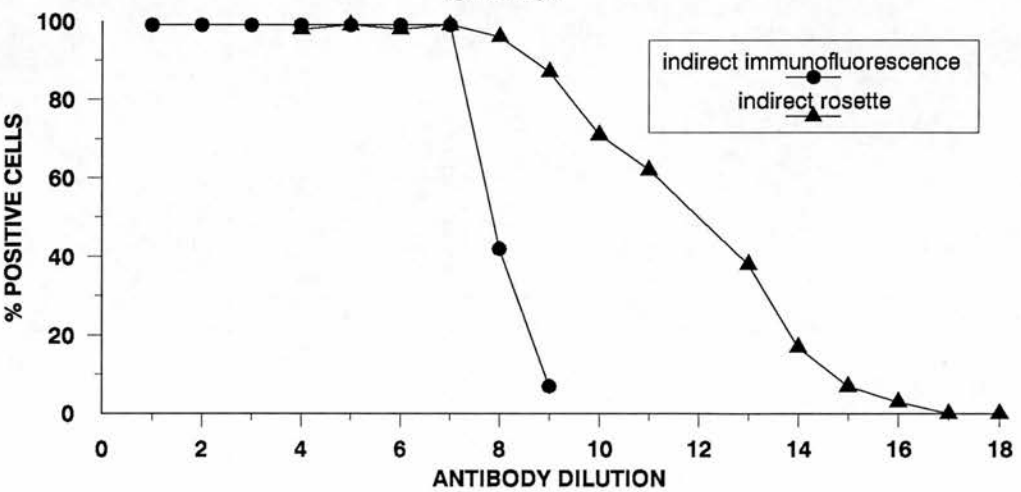
titration values obtained from the two techniques, the sensitivities of the two assay methods are comparable with respect to the 100% values. However, the rosette technique was more sensitive at higher dilutions of antibody. In view of the weaker expression of common leucocyte antigen on U937 cells, U937 cells coated with a dilution of BK19.45 antibody which was on the plateau portion of the titration curve obtained by indirect rosetting were used to monitor the efficiency of subsequent batches of sheep erythrocytes coated with antibody to mouse IgG. Batches of sheep erythrocytes which gave 100% rosettes in this test system were acceptable for use in subsequent rosette depletion experiments.

FIGURE 16. TITRATION OF THE MONOCLONAL ANTIBODY BK19.45 AGAINST U937 CELLS.



Tissue culture supernatant was titrated by using the indirect immunofluorescence and indirect rosette techniques.

FIGURE 17. TITRATION OF THE MONOCLONAL ANTIBODY BK19.45 AGAINST PERIPHERAL BLOOD MONONUCLEAR CELLS.



Tissue culture supernatant was titrated by using the indirect immunofluorescence and indirect rosette techniques.

To remove normoblasts and monocytes/macrophages from fetal liver mononuclear cells, these cells were coated with Ret40F and 61D3 monoclonal antibodies and the rosettes formed following incubation with antibody-coated sheep red cells were removed by centrifugation through ficoll hypaque. Antigen negative cells which remained at the ficoll interface were harvested for subsequent analysis. The average of the percentage of fetal liver mononuclear cells rosetting when a combination of the two antibodies was used was 28.2% (95% CI = 22.1-34.2; and range = 2-58). This value for the combined percentages of normoblasts and macrophages agreed well with the percentage of these cells as identified by their morphology after staining cytocentrifuged preparations with May-Grunwald-Giemsa (mean value = 29.6%; Table 12). After rosette depletion, the combined percentage of cells rosetting after relabelling with the monoclonal antibodies Ret40F and 61D3 was 2.4% (Table 11). Similarly, the combined percentages of normoblasts and macrophages observed in MGG stained cytocentrifuged preparations of rosette depleted populations were 0.1% (Table 12). These data show that the rosette depletion method was highly successful in removing normoblasts and monocytes/macrophages from fetal liver mononuclear cells. Plate 1 illustrates samples of MGG stained cytocentrifuged cell populations before and after rosette depletion with the Ret40F and 61D3 monoclonal antibodies.

The average yield of cells obtained from each liver after rosette depletion was 3.6×10^6 cells which represents 13.2% of the initial population of fetal liver mononuclear cells (Table 11). In general the final yield of cells related to the number of starting cells used in rosette depletion experiments. The cells obtained following rosette depletion were consistently viable (mean viability value = 95%; 95% CI = 94 - 96; range = 90 - 99).

The nature of the rosette depleted population and differences from the starting population, as observed by staining cytocentrifuged preparations of cells with MGG, are shown in Table 12. Following the successful removal of normoblasts and monocytes/macrophages, the percentage of undifferentiated blast cells increased from 48.6% to 76.5%. The remaining contaminating cells were cells at the promyelocyte to

Table 11. Cell yields following depletion of Ret40F and 61D3 positive cells from fetal liver mononuclear cells.

	Total cell number			% rosetting	% yield		
Separation stage[1]	Mean	(95% CI)	Range	Cells [2]	Mean	(95% CI)	Range
Post ficoll separation	3.1×10^7	(2.6-3.6)	5.4-58.0	28	100	-	-
Post rosette depletion	3.6×10^6	(2.7-4.4)	1.5-9.6	2 [3]	13.2	(9.4-16.9)	5-38

1. Number of experiments = 21.
2. Combined % of cells rosetting with 61D3 and Ret40F antibodies.
3. Data from 7 experiments: mean (±SEM) = 2.4 (0.5).

neutrophil stages of differentiation (8.6%), promonocytes (4.6%), cells with the morphological appearance of lymphocytes (7.9%) and unidentified cells (2.3%). The numbers of mature T (CD3 positive) and B (CD22 positive) lymphocytes were enumerated by APAAP staining in 4 experiments. The numbers of combined CD3(T) and CD22(B) cells, mean (+SEM) were 2.0 (+0.4) before rosette depletion. Therefore mature lymphocytes were present at a low frequency in the rosette depleted population. The purified undifferentiated blast cell population exhibited variable morphological features (Plate 1b). The nuclear chromatin pattern was generally open with usually multiple nucleoli and the nuclear outline varied from round to irregular convoluted forms. Cytoplasm was either scanty or moderate in quantity, weakly to strongly basophilic and sometimes vacuolated. Azurophilic granulation was absent. A more detailed characterisation of these cells is presented in Chapter 12.

Table 12. MGG differential of fetal liver cells before and after Ret40F and 61D3 rosette depletion.

	Purification stage [1]					
	before rosette depletion			after rosette depletion		
MGG differential (%)	Mean	(95% CI)	Range	Mean	(95% CI)	Range
Normoblasts	27.4	(21.0-33.7)	4-68	0	0	0
Lymphocytes [2]	8.9	(6.9-10.8)	2.5-17.5	7.9	(5.2-10.6)	0-24
Monocytes/macrophages	2.2	(1.0-3.4)	0 - 8	0.1	(0-0.2)	0.1
Others	2.8	(1.7-3.8)	0 - 8.5	2.3	(1.5-3.2)	0-8.5
Blasts	48.6	(43.6-53.7)	28-75	76.5	(76.5-79.5)	62-89.8
Promyelocytes/myelocytes/ metamyelocytes	9.6	(7.3-12.0)	0.25-20	7.6	(5.0-10.2)	1 - 2
segmented forms	0.5	(0.2-0.9)	0 - 2.8	1.0	(0.3-1.7)	0 - 7
Promonocytes [3]	-	-	-	4.6	(2.6-6.5)	0-13.5

1. Number of experiments = 20
2. Cells with morphological appearance of lymphocytes. CD3(T) and CD22(B) positive cells were determined by APAAP staining in 4 experiments. Combined CD3 and CD22 cells: mean(±SEM) % a) post ficoll separation = 2.0(0.4); b) post rosette depletion = 4.0(1.1).
3. Included under promyelocytes. Determined separately in 12 experiments: mean (±SEM) = 5.0 (0.7).

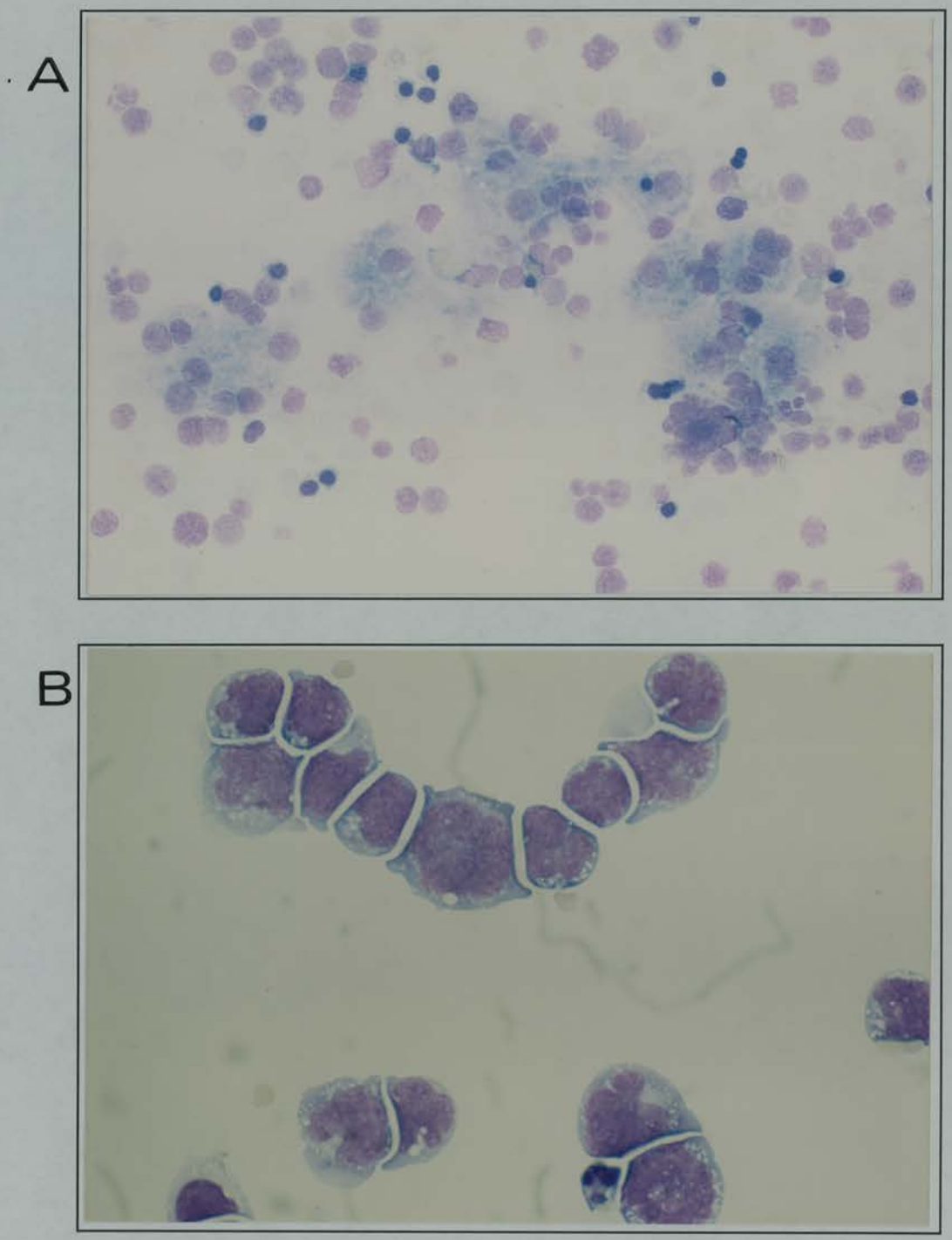


Plate 1. Photomicrographs of cells purified from human fetal liver following mechanical disruption of tissue. Plate 1a shows cells following ficoll separation where erythoblasts and macrophages are prominent (MGG x 500) and Plate 1b shows undifferentiated blast cells prepared following erythroblast and macrophage depletion (MGG x 625).

11.2 Purification of undifferentiated blast cells from human fetal liver following enzyme digestion of tissue, ficoll separation and erythroblast and macrophage depletion.

To enhance the yield of undifferentiated blast cells obtained from each fetal liver the purification procedure was modified. The new method incorporated initial enzyme digestion of fetal liver tissue, subsequent ficoll-hypaque separation of light density cells, which was performed twice, and finally rosette depletion of fetal liver mononuclear cells using the Ret40F and 61D3 monoclonal antibodies (see materials and methods). The total yield of cells obtained at each stage of the purification procedure is shown in Table 13. The average value for the yield of cells obtained following collagenase, hyaluronidase and dispase digestion of whole fetal liver was 2.15×10^9 cells. The nature of the cell population obtained following enzyme digestion of fetal liver, as identified from the differential of MGG stained cytocentrifuged preparations, is shown in Table 14. Following enzyme digestion the cell population was found to consist predominantly of erythroblasts (84.8%), which were mainly at intermediate to late stages of development (Plate 2a). Other populations were minor and consisted of monocytes and macrophages (5.7%), cells with the morphological appearance of lymphocytes (2.5%), undifferentiated blast cells (5.2%), promonocytes and cells at the promyelocyte to neutrophil stages of maturation (0.6%) and unidentified cells (1.2%). Following the first ficoll-hypaque fractionation step the average of values obtained for the yield of cells was 1.07×10^9 cells, which represented 49.8% of the cell population obtained following enzyme digestion of whole fetal liver (Table 13). This number of cells obtained was far greater than the yield of cells mechanical disruption of tissue and ficoll-hypaque separation of mononuclear cells (3.8×10^7, Table 10). The differential of cells obtained after the first ficoll separation did not differ significantly from that of the cells obtained following enzyme digestion of fetal liver. After separating the mononuclear cell population from the enzyme digest of fetal liver, the percentage of normoblasts fell slightly from 84.8% to 80.9%. In association, there was a marginal increase in the percentages of other minor cell populations (Table 14). The normoblasts obtained following ficoll-hypaque fractionation of the enzyme digest of fetal liver

Table 13. Cell yields following purification of fetal liver cells by enzyme digestion, ficoll separation and depletion of erythroblasts and macrophages.

	Total Cell Number			% Rosetting	% Yield	
Purification stage[1]	Mean	(95% CI)	Range	Cells[2]	Mean	(95% CI)
Post enzyme digestion[3]	2.2×10^9	(1.72-2.57)	0.75-4.53	-	100.0	-
Post 1st ficoll separation	1.1×10^9	(0.81-1.34)	0.12-2.30	-	49.8	(36.8-60.9)
Post 2nd ficoll separation	2.2×10^8	(0.99-3.36)	0.3 -14.0	54.0	10.1	(4.5-15.3)
Post rosette depletion	7.3×10^6	(4.2-10.4)	2.7-17.3	ND[4]	0.34	(0.2-0.47)

1. Number of experiments = 23; rosette depletion data from 12 experiments.
2. Combined % of cells rosetting with 61D3 and Ret40F antibodies.
3. Tissue was digested by using a mixture of collagenase, dispase and hyaluronidase.
4. ND = not determined.

Table 14. MGG differentials of fetal liver cells purified following enzyme digestion, ficoll separation and depletion of erythroblasts and macrophages.

	Purification stage							
	Enzyme digestion[2]		1st Ficoll		2nd Ficoll		Rosette depletion	
MGG differential (%)[1]	Mean	(+95% CI)	Mean	(+95% CI)	Mean	(+95% CI)	Mean	(+95% CI)
Normoblasts	84.8	(3.9)	80.9	(4.8)	52.2	(8.8)	0.3	(0.3)
Lymphocytes[3]	2.5	(0.9)	3.5	(1.5)	6.2	(2.1)	11.9	(4.4)
Monocytes/macrophages	5.7	(1.4)	5.1	(1.6)	11.3	(3.0)	0.5	(0.4)
Others	1.2	(0.9)	1.6	(0.6)	3.7	(1.4)	5.5	(1.6)
Blasts	5.2	(1.6)	8.2	(3.0)	24.3	(6.5)	59.2	(4.8)
Promyelocytes/myelocytes/ metamyeloctyes	0.5	(0.4)	0.6	(0.5)	2.0	(1.6)	8.3	(4.3)
Segmented forms	0.1	(0.1)	0.1	(0.1)	0.3	(0.2	0.4	(0.4)
Promonocytes[4]	ND		ND		ND		13.9	(2.5)

1. Number of experiments = 23; rosette depletion data from 12 experiments.
2. Tissue was digested by using a mixture of collagenase, dispase and hyaluronidase.
3. Cells with morphological appearance of lymphocytes.
4. ND = not determined separately, included under promyelocytes.

were similar in morphology, being predominantly at intermediate to late stages of development (Plate 2b).

Following the second ficoll-hypaque fractionation step there was a large fall in cell yield. The average of values obtained for the yield of cells was 2.17×10^8 cells, which represented 10.1% of the initial population obtained following enzyme digestion of fetal liver (Table 13). In association with the fall in cell yield, there was a change in the nature of the cell population as identified by the differential of cells seen in MGG stained cytocentrifuged preparations. After the first ficoll-hypaque fractionation step, the average of values obtained for the percentage of normoblasts was 80.9%. In contrast, after fractionation of cells for a second time over ficoll-hypaque, the average of values obtained for the percentage of normoblasts was reduced to 52.2% (Table 14). Furthermore, the normoblasts present after the first and second ficoll separations were qualitatively different. After the second ficoll separation there were higher proportions of pronormoblasts and normoblasts at early stages of development (Plate 2c) and the morphological discrimination between undifferentiated blast cells and early erythroid precursors was more difficult. In association with the fall in the normoblast population, there was an increase in other cell populations, particularly undifferentiated blast cells (24.3%), monocytes and macrophages (11.3%) and cells with the morphological appearance of lymphocytes (6.2%) (Table 14). At this stage, following the second ficoll fractionation and prior to the rosette depletion procedure, the number of cells obtained (2.17×10^8 cells, Table 13) remained far greater than the yield of cells obtained following mechanical disruption of tissue and ficoll separation of mononuclear cells (3.8×10^7 cells, Table 10).

After fractionation of fetal liver cells over ficoll-hypaque for the second time, the cells were depleted of normoblasts and macrophages by the indirect rosette technique after coating cells with the monoclonal antibodies Ret40F and 61D3 as previously described. The data from 12 experiments in which the rosette depletion procedure was performed is shown in Table 14. After the second ficoll fractionation, the percentage of cells rosetting when a combination of the two depleting antibodies

Ret 40F and 61D3, used was 54%. This value showed good agreement with that obtained for the combined percentages of normoblasts and macrophages, as identified by their morphology on MGG stained cytocentrifuged preparations which was 63.5% (Table 14). Following rosette depletion, normoblasts and macrophages were successfully removed as shown by the combined percentages of these cells observed in MGG stained cytocentrifuged preparations of rosette depleted mononuclear cells (0.8%, Table 14).

The average value for the yield of cells obtained from each liver after rosette depletion was 7.3×10^6 cells, which represented 0.34% of the initial population of cells obtained following enzyme digestion. These cells were consistently viable. As in the case of the previous purification method, the yields of cells obtained at each stage of the purification procedure related in general to the number of starting cells. Using the modified method incorporating enzyme digestion of liver tissue, where the number of starting cells was higher, the average final yield of cells obtained following rosette depletion (7.6×10^6 cells, Table 4) was greater than that obtained by using the original method of mechanical disruption of tissue (3.6×10^6 cells, Table 11).

The nature of the rosette depleted population, as observed by the MGG differential of cytocentrifuged preparations, is shown in Table 14. The purified cell population consisted mainly of undifferentiated blast cells (59.2%) which exhibited a morphology (Plate 2d) similar to that described for rosette depleted cells generated using the previous method (Plate 1b). The remaining cells consisted of promonocytes (13.9%), cells at the promyelocyte to neutrophil stages of differentiation (8.7%), cells with the morphological appearance of lymphocytes (11.9%) and unidentified cells (5.5%).

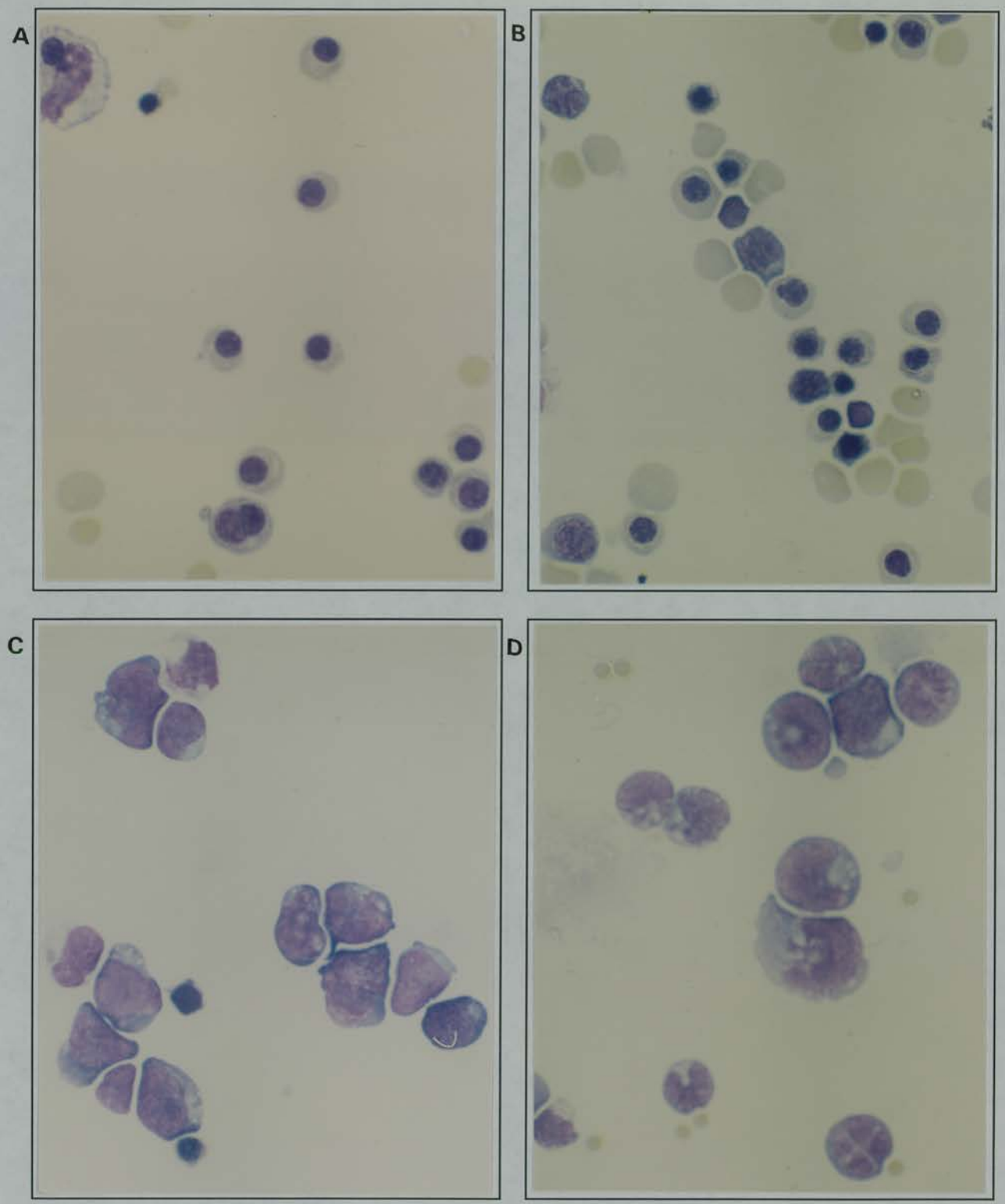


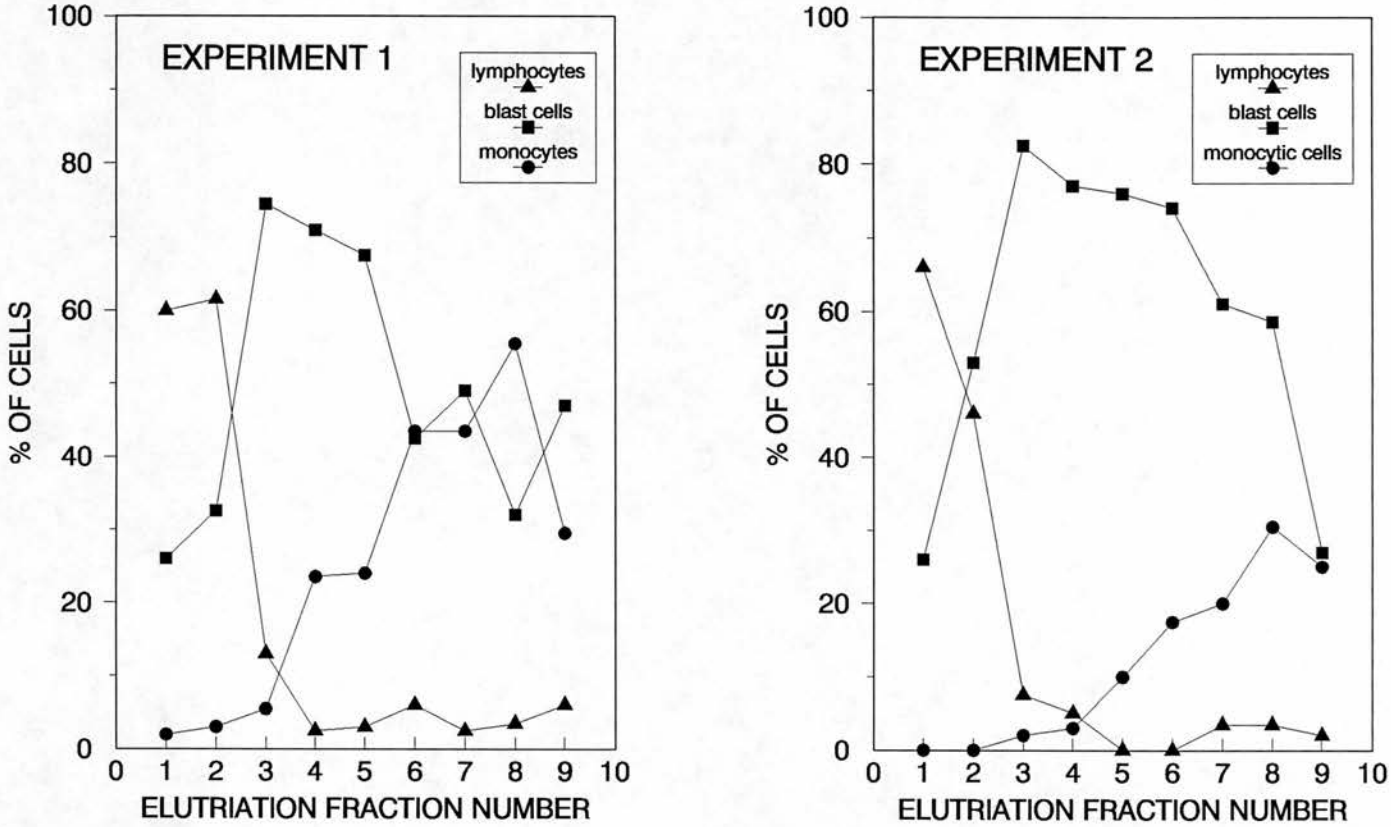
Plate 2. Photomicrographs of cells purified from human fetal liver following enzyme digestion of tissue. Mature erythroblasts predominate following enzyme digestion (a) and first ficoll separation (b). Following second ficoll separation (c) early erythroblasts and undifferentiated blasts predominate and following erythroblast and macrophage depletion (d) undifferentiated blasts and promonocytes are the dominant cell populations. (MGG x 500).

11.3 Further purification of the undifferentiated blast cell population by cell elutriation.

The blast cells purified following enzyme digestion of fetal liver tissue and depletion of erythroblasts and macrophages from a mononuclear cell fraction were themselves heterogeneous in nature. There was also a significant contaminating population of promonocytes. To attempt to obtain a more homogeneous blast cell population and to eliminate contaminating promonocytes, the above cells were further purified by cell elutriation. This procedure separates cells according to their size and buoyant density. In two experiments, fractions were collected at incremental flow rates of 4.7, 9.0, 13.3, 17.8, 22.0, 26.3, 30.4 and 34.5 mls/minute and the rotor off fraction was also collected.

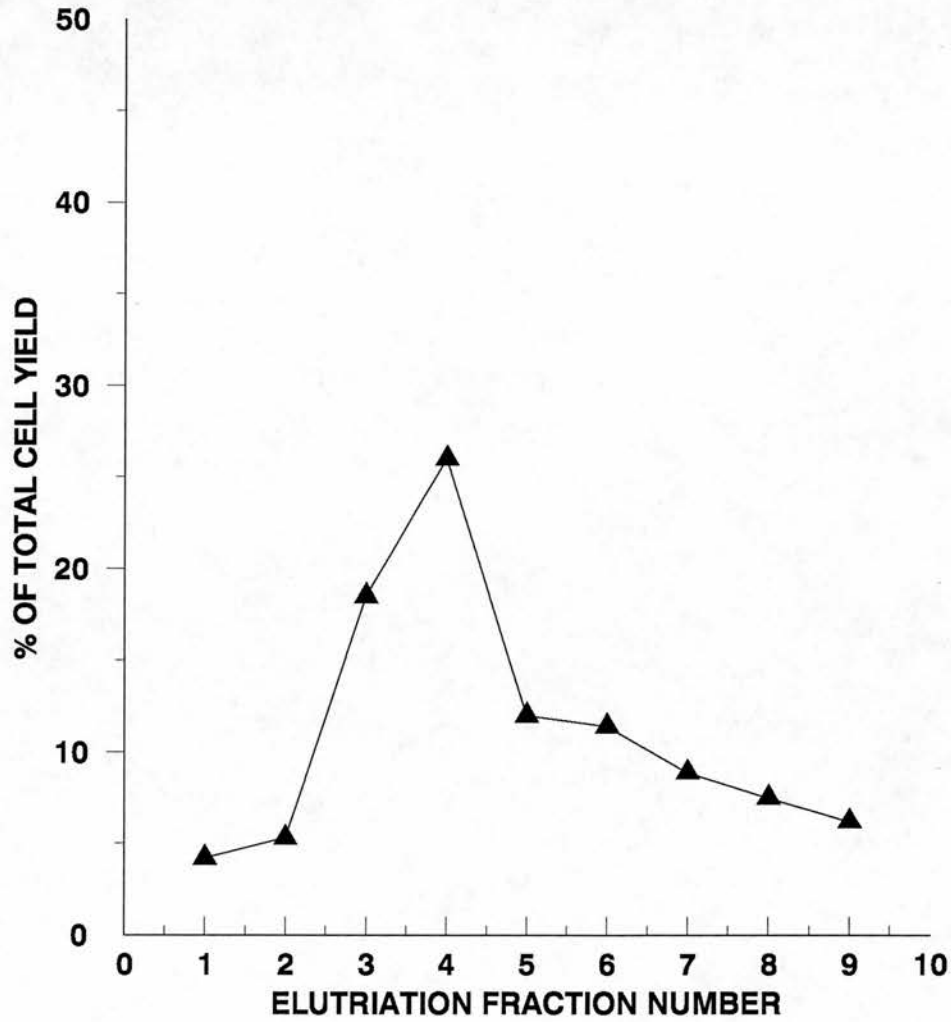
The nature of the cell population present within each elutriation fraction, as observed by the MGG differential of cytocentrifuged preparations, is shown in Tables 15a and 15b and the changes in major cell populations with respect to elutriation fraction number are charted in Figure 18. The morphology of cells obtained from each elutriation fraction is illustrated in Plate 3 a-i. The first two fractions obtained at flow rates of 4.7 ml/minute and 9.0 ml/minute, were dominated by cells with the morphological appearance of lymphocytes. These cells were not present to any large degree in subsequent fractions (<13%). The third fraction, obtained at a flow rate of 13.3 ml/minute, contained the largest percentage of blast cells which were present at levels of 82.5% and 74.5%. The percentage of blast cells remained high in the next two fractions which were obtained at flow rates of 17.8 ml/minute and 22.0 ml/minute. In subsequent fractions the percentage of blast cells declined although the rate of fall was more gradual in experiment number 2 (Figure 18). As regards monocytic cells, which were mainly promonocytes, they were present to a small degree in the first two fractions obtained at flow rates of 4.7 ml/minute and 9.0 ml/minute (< 3%). In subsequent fractions there was a progressive increase in the percentage of monocytic cells and in the fraction obtained at a flow rate of 30.4 ml/minute 30.5% and 55.5% of the cells were monocytic.

FIGURE 18. SEPARATION OF BLAST CELLS BY CELL ELUTRIATION



The figures show the separation of predominant cell populations which were lymphocytes, blast cells and promonocytes.

FIGURE 19. NUMBERS OF CELLS OBTAINED IN EACH ELUTRIATION FRACTION.



The data are presented as a percentage of the total cell yield and are the mean of values obtained from two experiments.

Table 15a. MGG differential of cells obtained in elutriation fractions (experiment 1).

	Elutriation Fraction								
MGG differential %	4.7	9.0	13.3	17.8	22.0	26.3	30.4	34.5	Rotor Off
Normoblasts	0	0	0	0	0	0	0	0	13
Lymphocytes[1]	60	61.5	13	2.5	3	6	2.5	3.5	6
Monocytic cells[2]	2	3	5.5	23.5	24	43.5	43.5	55.5	29.5
Others	10	2	6	3	2.5	0.5	2.5	1	3.5
Blasts	26	32.5	74.5	71	67.5	42.5	49	32	47
Promyelocytes Myelocytes/metamyelocytes	2	1	0.5	0	3	7.0	1.5	8	1
Segmented band forms	0	0	0.5	0	0	0.5	0.5	0	0

1. Cells with morphological appearance of lymphocytes.
2. Promonocytes/monocytes/macrophages.

Table 15b. MGG differential of cells obtained in elutriation fractions (experiment 2)

	Elutriation fraction								
MGG differential (%)	4.7	9.0	13.3	17.8	22.0	26.3	30.4	34.5	Rotor Off
Normoblasts	0	0	0	0	0	0	0	0	36
Lymphocytes[1]	66	46	7.5	5	0	0	3.5	3.5	2
Monocytic cells[2]	0	0	2	10	17.5	20	22	30.5	25
Others	8	1	4.5	7	3.5	4.5	11	3.5	6
Blasts	26	53	82.5	77	76	74	61	58.5	27
Promyelocytes/myelocytes metamyelocytes	0	0	3.5	1	2.5	1.5	2.5	4	3
Segmented band forms	0	0	0	0	0.5	0	0	0	1

1. Cells with morphological appearance of lymphocytes
2. Promonocytes/monocytes, macrophages.

Plate 3. Cells purified from human fetal liver after elutriation of erythroblast and macrophage depleted mononuclear cells.

Illustrations are photocopies of colour photomicrographs of cells stained with May-Grunwald-Giemsa (magnification x 500). Letters a - h represent sequential elutriation fractions from 1 - 8 and i represents the rotor off fraction.

a
b
c
d
e
f
g
h
i

The distribution of cell numbers within each elutriation fraction was also determined. As in the case of the differential data the results obtained from both experiments were similar and are therefore presented as mean values in Figure 19. Optimal separation of lymphocytes, blast cells and monocytic cells was observed at flow rates of 13.3 ml/minute and 17.8 ml/minute. These two fractions also contained the largest numbers of cells (fraction Nos. 3 and 4).

The first two fractions obtained at flow rates of 4.7 ml/minute and 9.0 ml/minute contained 9.6% of the total cells recovered. Almost half of the total cells recovered (44.5%) were present within the next two fractions obtained at flow rates of 13.3 ml/minute and 17.8 ml/minute. Progressively smaller numbers of cells (12-6%) were present in the fractions obtained at flow rates >22.0 ml/minute. When purified blast cells were used in subsequent experiments the fractions obtained at flow rates 13.3 ml/minute and 17.8 ml/minute were combined. The yields of cells from the two experiments shown were 1.1×10^6 cells and 0.33×10^6 cells. The data show that elutriation of cells at flow rates of 13.3 ml/minute and 17.8 ml/minute (fraction Nos. 3 and 4) gave rise to an optimal combination of cell yield and separation of undifferentiated blast cells from other cell types. Cells obtained when these two fractions were pooled were subjected to further characterisation (Chapter 12) and were used in further studies (Chapters 14, 15).

Therefore in a further five experiments cells collected only at flow rates of 13.3 ml/minute and 17.8 ml/minute were pooled and subjected to further analysis. These data for the yields of cells and the percentage differentials in the five experiments are shown in Table 16. The average of values obtained for the yield of cells in these two fractions was 1.75×10^6 cells. This number of cells represented 0.07% of the starting number of cells obtained following enzyme digestion of fetal liver. For these five same experiments the average of the values obtained for the yield of cells (+SEM) following rosette depletion was 8.7×10^6 (+3.0). Therefore 20% of the rosette depleted cells were recovered following collection of the two elutriation fractions. The dominant cell type within these pooled fractions was undifferentiated blast cells which accounted

for 70% of cells. Cells with morphological appearance of lymphocytes accounted for 15% of cells and monocytic cells (mainly promonocytes) accounted for 11% of cells.

Table 16 Numbers of cells obtained and the percentages of blast cells within fractions elutriated at flow rates of 13.3 and 17.8ml/minute.

Experiment[1].	Cells		MGG differential (%)		
	Total No. x 10^6	% yield	blasts	promonocytes	lymphocytes
1	3.27	0.08	76	14	7
2	0.88	0.07	82	14	0
3	0.53	0.03	60	11	25
4	1.04	0.05	69	10	18
5	3.04	0.12	62	8	24
Mean	1.75	0.07	69.8	11.4	14.8
(SEM)	(0.58)	(0.02)	(4.2)	(1.2)	(4.9)

1. Cells were elutriated at flow rates of 13.3 ml/minute and 17.8 ml/minute and these fractions were then pooled for analyses.

Comparison of the morphological appearances of the blast cells obtained after cell elutriation with those obtained by merely depleting normoblasts and macrophages from fetal liver mononuclear cells revealed that the cell elutriated blast cells were more homogeneous in nature and present at a higher percentage (Table 14, Table 16). The blast cells were more uniform as regards their size, nuclear morphology and cytoplasmic characteristics. These points are illustrated in Plates 3c-d and Plate 2d.

CHAPTER 12 CHARACTERISATION OF UNDIFFERENTIATED BLAST CELLS PURIFIED FROM HUMAN FETAL LIVER.

12.1 Characterisation of blast cells obtained following depletion of normoblasts and macrophages from fetal liver mononuclear cells.

The blast cells obtained by depleting normoblasts and macrophages from fetal liver mononuclear cells separated following mechanical disruption of tissue, were characterised as to their cytochemistry, expression of cell surface antigens, their ability to form colonies when plated in semi-solid medium and for the percentage of cells in each phase of the cell cycle.

12.1. Cytochemical investigations

Cytocentrifuge preparations of blast cells were stained to reveal the extent of their Sudan Black B and periodic acid-Schiff positivity and whether they expressed the enzymes chloroacetate esterase (granulocyte specific) and butyrate esterase (monocyte specific). The results are shown in Table 17 and MGG differential data from the same experiments are also shown for comparison.

The number of butyrate (monocyte specific) esterase positive cells was 3% and this value correlated well with the number of monocytic cells identified following staining with MGG (4%). The number of chloroacetate esterase (granulocytic specific) positive cells was 11% and this value also agreed well with the percentage of granulocytic cells identified following staining with MGG (11%). The value for Sudan Black B positivity, which is not lineage specific and which is present in more mature granulocytic and monocytic cells, was 16%. This value correlated well with the combined percentages of monocytic and granulocytic cells identified following staining with MGG (15%). The value for periodic acid-Schiff positivity was 12% and the staining was of a weak, diffuse nature. This marker is seen in myeloid cells showing evidence of maturation and is particularly strong in mature granulocytes. This degree and nature of PAS positivity was consistent with the extent of myeloid maturation observed in MGG stained preparations.

Undifferentiated blast cells were clearly seen to be negative for Sudan Black B, chloroacetate esterase, butyrate esterase and periodic acid-Schiff reagent. Plate 4 illustrates the cytochemical staining observed.

Table 17 Cytochemical investigations on blast cells obtained following depletion of erythroblasts and macrophages from fetal liver mononuclear cells.

Cytochemical stain	% cells positive Mean	(±SEM)
Sudan Black B	16	(3.0)
Chloroacetate esterase	11	(1.8)
Butyrate esterase	3	(1.8)
Periodic acid -Schiff	12	(4.5)
MGG differential (%)		
Normoblasts	0	(0)
Lymphocytes	4	(2.0)
Blasts	79	(0.9)
Monocytic cells	4	(0.9)
Granulocytic cells	11	(2.9)
Others	2	(0.6)
No. of experiments = 3		

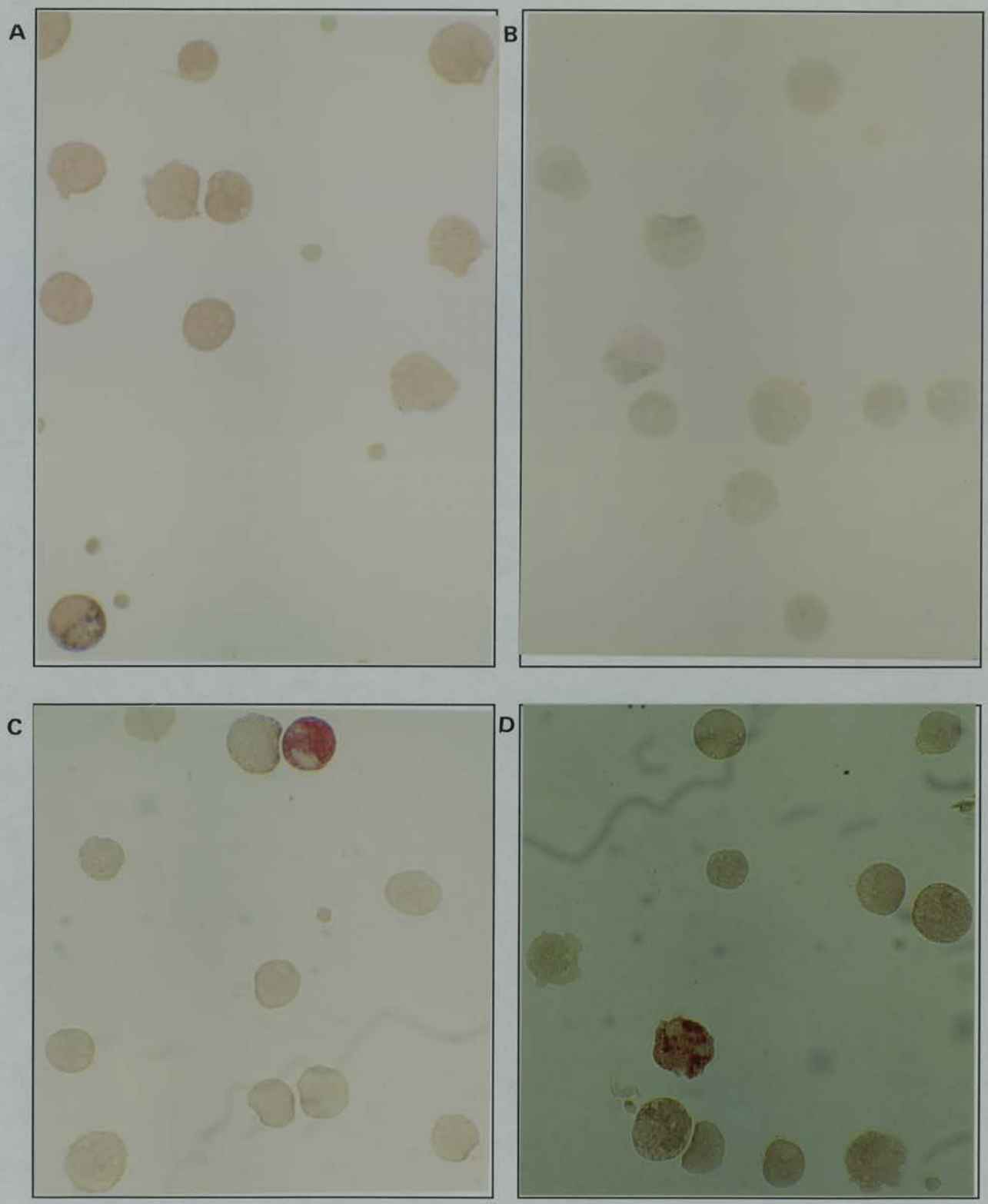


Plate 4. Illustration of cytochemical staining of blast cells purified from human fetal liver following erythroblast and macrophage depletion. Plate 4a Sudan Black B; Plate 4b Periodic acid-Schiff; Plate 4c Chloroacetate esterase and Plate 4d Butyrate esterase.(mag x 500). Undifferentiated blast cells were negative for these markers.

12.1.2 Immunophenotypic analysis.

Cells purified from human fetal liver following erythroblast and macrophage depletion were assessed for their expression of cell surface antigens known to be present on haemopoietic cell populations. These studies were performed using a combination of techniques including direct and indirect rosetting, indirect immunofluorescence and the APAAP technique. The results are shown in Table 18. The percentage of cells expressing common leucocyte antigen was 99% which confirmed the haemopoietic nature of the purified cell population. The percentage of cells expressing glycophorin C, an erythroid antigen, was low (1%) illustrating the efficiency of the erythroblast depletion procedure. The percentages of positive cells for the mature myeloid antigens CD11c, CD15 and CD14 were all low (<8%) and these values were consistent with the numbers of mature myeloid cells observed following staining with MGG (Chapter 11, Table 12). The number of mature T cells present, as identified by CD3 staining, was also low (1%). The antigen CD5, which identifies T cells and a subset of B cells, was also expressed at a low level of 4%. The mean value for the percentage of CD10 positive cells was 18% and the corresponding value for CD19 positive cells was 14%. The CD10 antigen is expressed on B cell progenitors and also weakly on mature granulocytes and the CD19 antigen is a marker for early B cells and persists throughout B cell differentiation. As the percentage of mature granulocytes in the purified population is small, these data indicate that B cell progenitors are present within the population purified. Very much larger percentages of cells were positive for HLA Class II (59%), CD7 (46%) and CD34 (32%) antigens. These antigens are known to be expressed on early haemopoietic cells. For example Class II HLA antigen expression is a feature of progenitor cells, B cells and macrophages. CD7 expression is a feature of T cell and myeloid precursor cells and CD34 expression is a feature of haemopoietic stem cells and committed progenitor cells. The presence of large numbers of HLA class II positive, CD7 positive and CD34 positive cells within the population purified is consistent with their largely undifferentiated nature as revealed by cytochemical analyses. The ability of this undifferentiated blast cell population to differentiate and the nature of their progeny were subsequently studied.

Table 18. Immunophenotypic analysis of undifferentiated blast cells obtained following depletion of erythroblasts and macrophages from fetal liver mononuclear cells.

Antigen	% cells positive[1] Mean	(±SEM)
Common leucocyte antigen	99	(0.5)
Erythroid		
glycophorin C	1	(0.4)
Myeloid		
CD11c	7	(0.8)
CD15	8	(1.0)
CD14	3	(1.3)
Lymphoid		
CD3 (T cell)	1	(0.6)
CD5 (T cell,B cell subset)	4	(1.5)
CD10 (B cell progenitor)	18	(3.3)
CD19 (B cell)	14	(7.0)
Others		
CD7 (T cell, myeloid precursors)	47	(6.3)
HLA class II (progenitors, B cells, macrophages)	59	(5.5)
CD34 (early cells)	32	(4.2)
Negative control[2]	2	(0.8)

1 = Data from two experiments.

2 = Cells were stained using a monoclonal antibody to rotavirus

12.1.3 Analysis of colony forming ability.

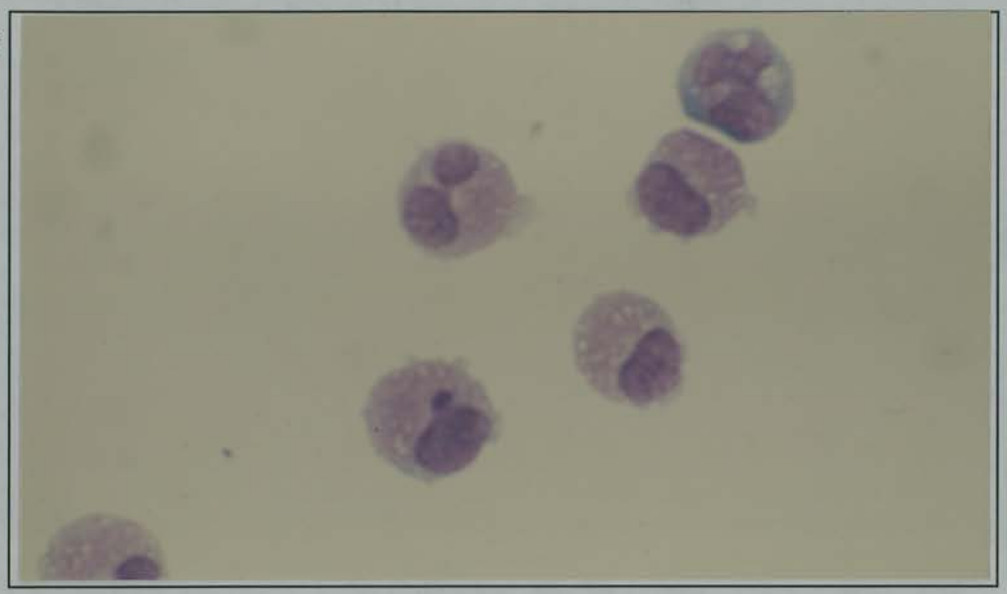
Cells, purified from human fetal liver following erythroblast and macrophage depletion, were plated into semi-solid medium to determine the numbers of GM-CFC and BFU-E present and to determine whether the blast cells themselves were able to give rise to blast cell colonies. The results are shown in Table 19. The average of values obtained for the numbers of GM-CFC per 10^5 cells of the mononuclear fraction was 152 and the corresponding value for BFU-E was 136. These values are higher than the normal reference ranges for adult bone marrow and are consistent with previously reported data for CFC numbers in studies of human fetal liver cell populations (Toksoz and Brown, 1984; Rowley et al 1978; Emerson et al 1985). Following depletion of erythroblasts and macrophages there was some enrichment of GM-CFC and BFU-E. Values obtained for the numbers of GM-CFC and BFU-E within the rosette depleted population were 244 per 10^5 cells and 237 per 10^5 cells, respectively. These numbers of colony forming cells represent 0.5% of the cell population. Therefore the large majority of the blast cells, themselves, did not give rise to colonies under the culture conditions used in these assays. Plate 5 illustrates examples of colony morphology and colony identification following analysis of cytocentrifuged preparations of individual colony contents.

Table 19. Assay of colony forming ability of cells purified from human fetal liver following erythroblast and macrophage depletion.

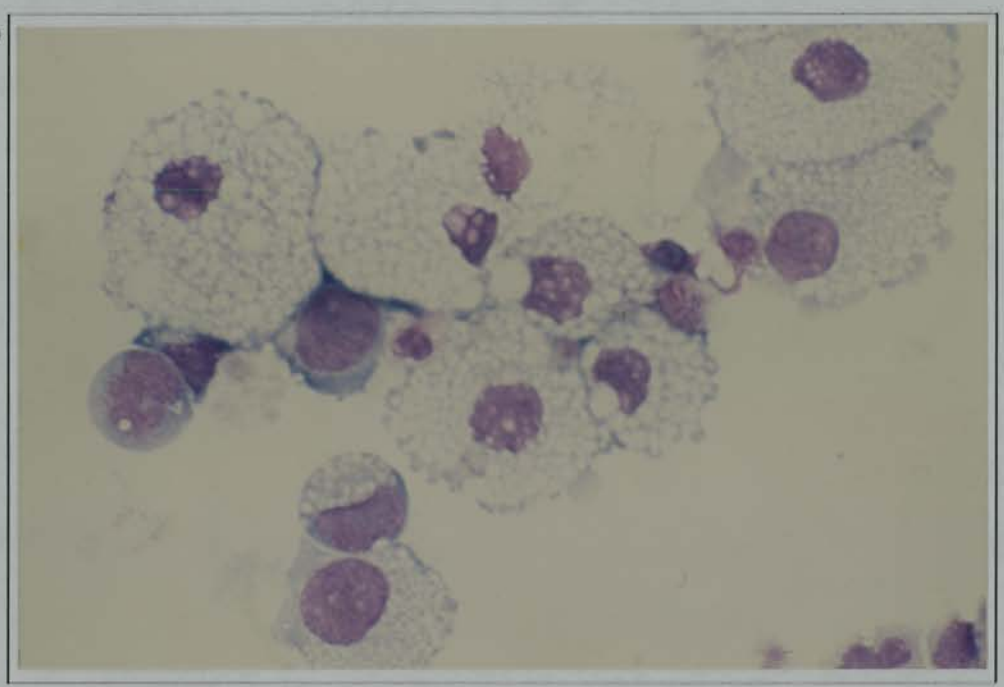
	No. of CFU per 10^5 cells	
	GM-CFC[1]	BFU-E[2]
Purification stage	Mean (±SEM)	Mean (±SEM)
Post ficoll separation	152 (50)	136 (23)
Post rosette depletion	244 (42)	237 (43)

1. Number of experiments = 4
2. Number of experiments = 3

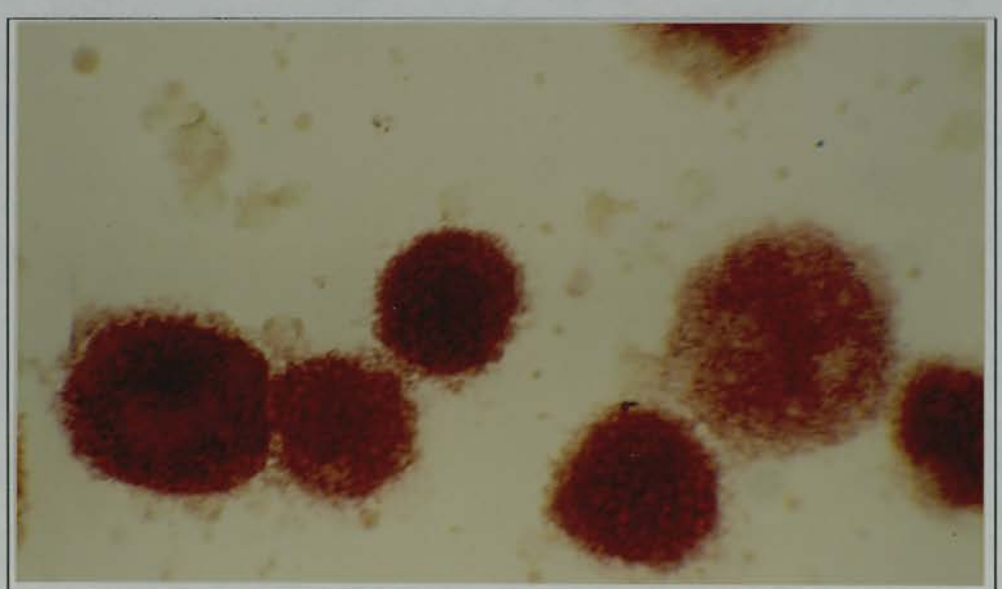
C



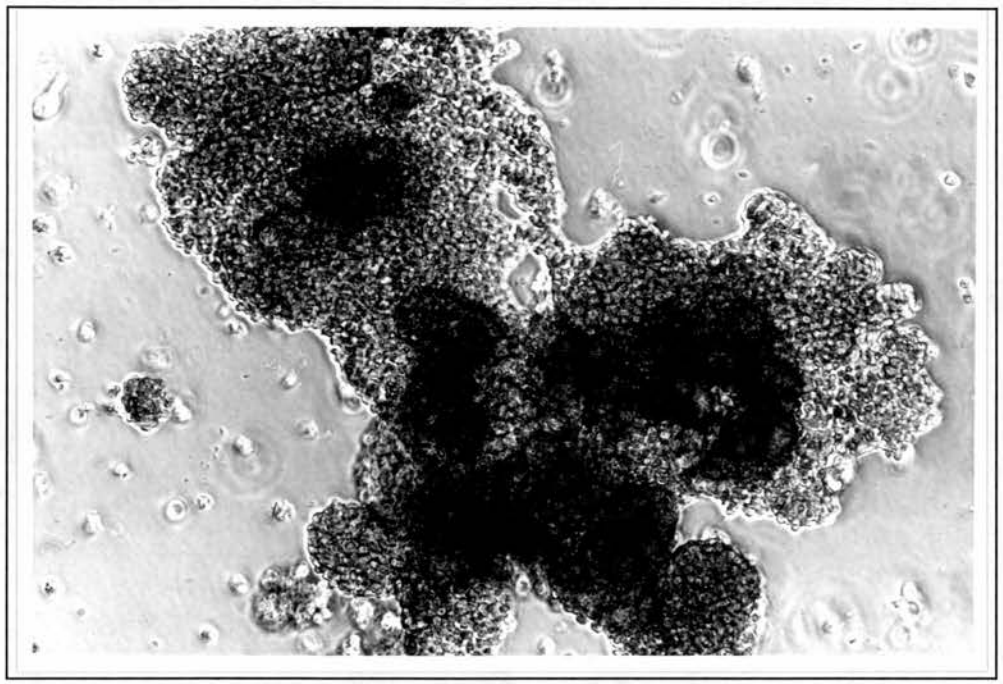
D



E



A



B

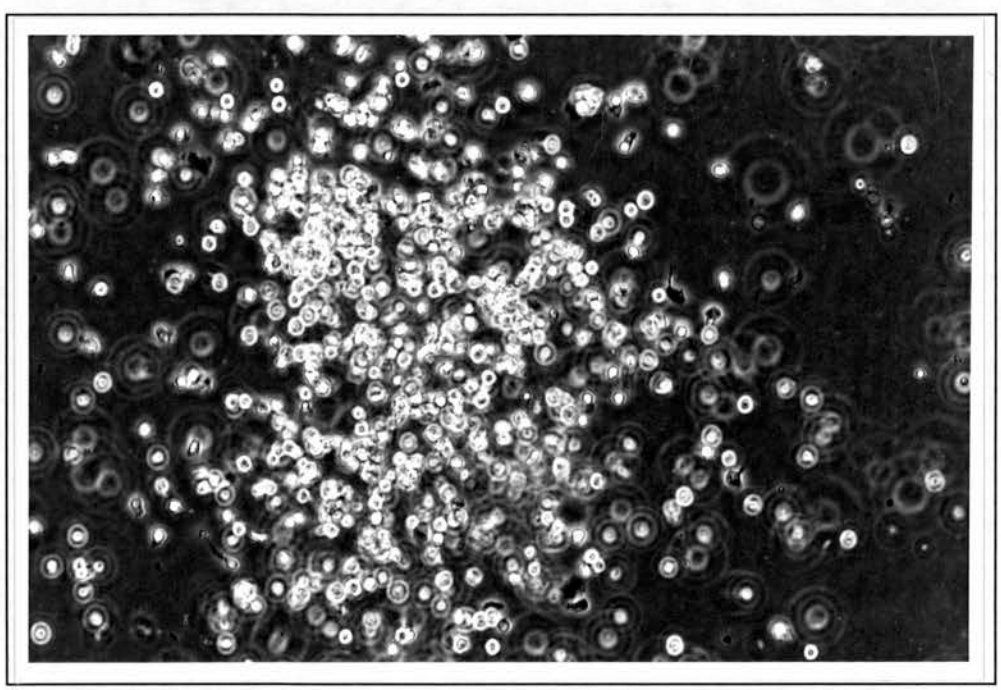


Plate 5. Photomicrographs of colony morphology of colonies grown from cells purified from human fetal liver . Plate 5a shows a BFU-E colony (x 140) and Plate 5b shows a granulocyte-macrophage colony (x 280). Plates 5c and 5d show MGG stained cytocentrifuge preparations of the colony contents of a granulocyte colony (5c) and a macrophage colony (5d) respectively. Plate 5e shows the contents of a macrophage colony stained for alpha-naphthyl acetate esterase (x 500).

12.1.4. Analysis of phase of cell cycle.

The percentage of the undifferentiated blast cell population at each phase of the cell cycle is shown in Table 20. The analysis was performed once and comparative studies were carried out on HL60 cells and tonsillar B lymphocytes. The corresponding plots of cell number versus DNA content are shown in Figure 20. The undifferentiated blast cell population purified from fetal liver exhibited an S phase population intermediate between that of tonsillar B cells and HL60 cells. It is apparent that the vast majority (81%) of the fractionated fetal liver cells were out of cell cycle.

Table 20. Analysis of phase of cell cycle of undifferentiated blast cell population purified from human fetal liver following Ret40F and 61D3 rosette depletion.

n = 1	Cell Type		
	Fetal liver blast cells	HL60 cells	Tonsillar B lymphocytes
Phase of cell cycle	% cell population	% cell population	% cell population
G_1	81	39.7	98.3
S	14.9	52.2	0.5
G_2/M	4.1	8.1	1.2

12.2. Characterisation of blast cells obtained after cell elutriation.

12.2.1 Cytochemical and immunocytochemical analysis

The numbers of cells available within each elutriation fraction were small and this therefore limited further analysis of the undifferentiated blast cell population. However, the presence of the surface antigens CD34 and CD10 were determined, using an indirect immunofluorescence technique, on cells within each elutriation fraction. The analysis of CD34 positive cells was performed twice. As in the case of MGG

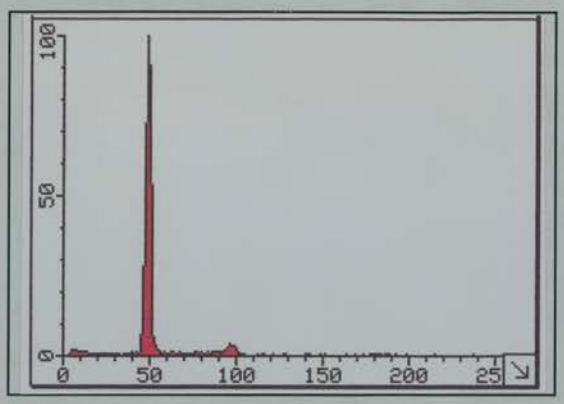


Figure20a.Undifferentiated myeloid blast cells purified from human fetal liver.

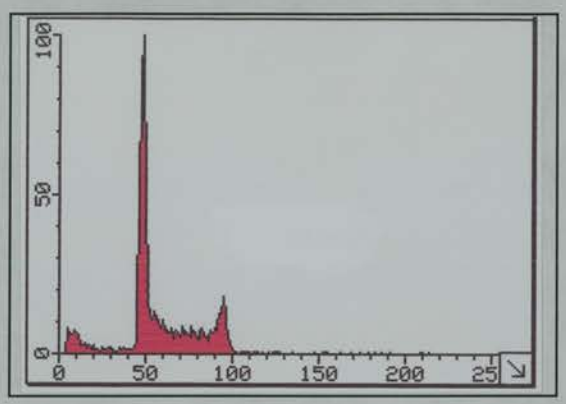


Figure 20b. HL60 cells.

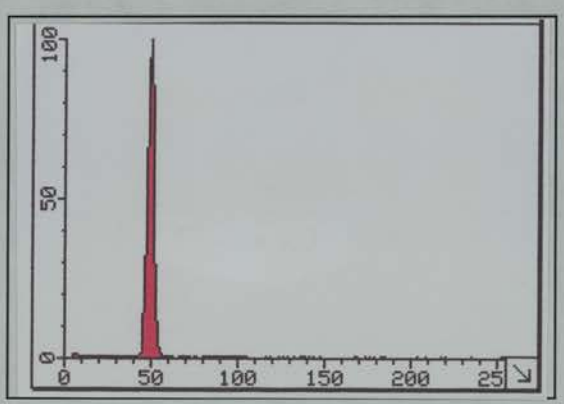


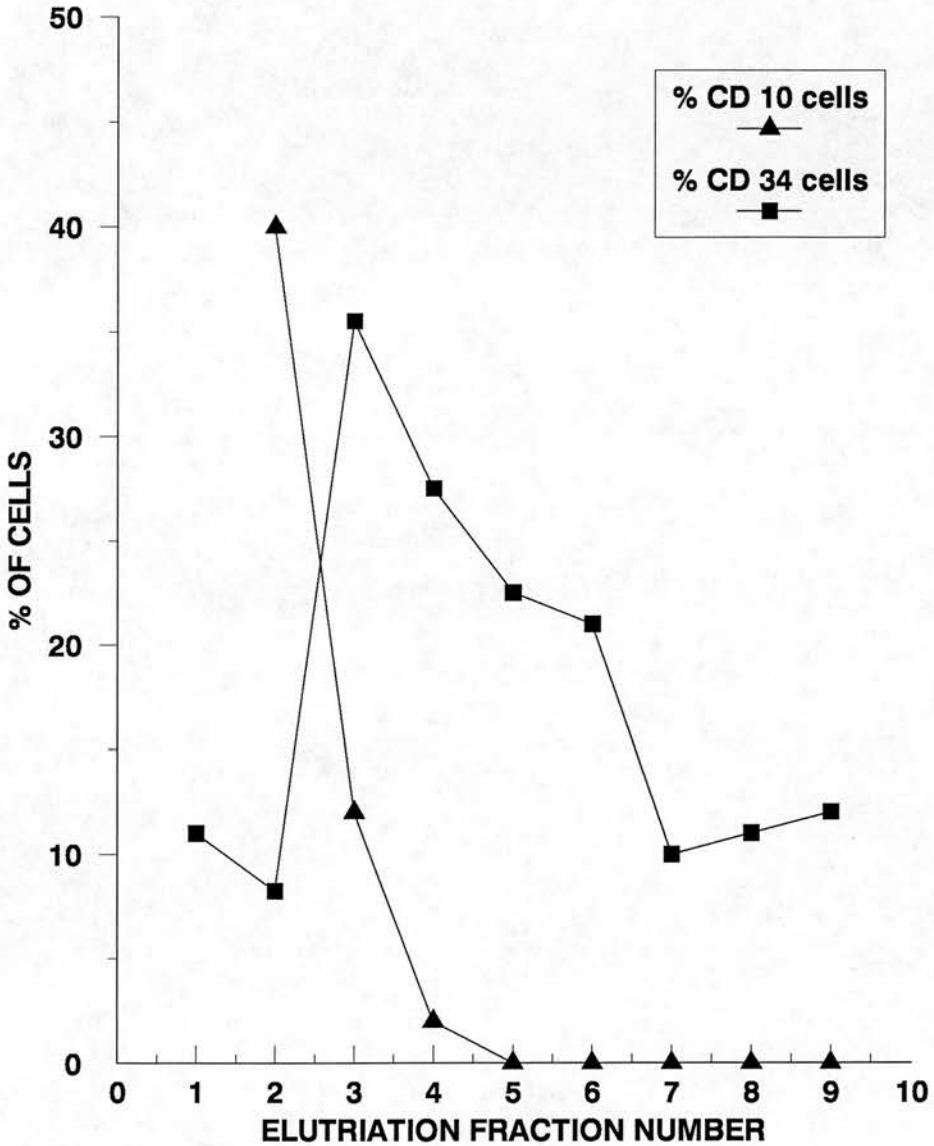
Figure 20c.Tonsillar B cells.

Figure 20. Cell cycle profiles of different cell populations. Test cells were processed using a commercial kit (Cycletest, Becton Dickinson, UK) and analysed in a flow cytometer (FACScan) using commercial software (Cellfit, Becton Dickinson, UK). Cell number on the vertical axis is plotted against fluorescence intensity (DNA content) on the horizontal axis. Undifferentiated myeloid blast cells exhibited an S phase population intermediate between that of tonsillar B cells and HL60 cells.

differential and cell yield data, the results for both experiments were similar and are therefore presented as the mean of values, in Figure 21. The analysis of CD10 positive cells was performed once and the results are also presented in Figure 21. The percentages of CD34 positive cells were low in the first two fractions obtained at flow rates of 4.7 ml/minute (11%) and 9.0 ml/minute (8%). CD34 positive cells were predominant in the next two fractions. In the fractions collected at flow rates of 13.3 ml/minute and 17.8 ml/minute 36% and 38% of the cells were CD34 positive, respectively. These fractions were also found to contain the highest percentages of undifferentiated blast cells and the largest cell numbers. In subsequent fractions the percentage of CD34 positive cells was lower and in the range 10-23% (Figure 21). When the data is expressed in terms of absolute numbers of CD34 positive cells, the yield of positive cells was also maximal in the fractions obtained at flow rates of 13.3 ml/minute and 17.8 ml/minute (fraction nos. 3 and 4). The percentage of CD10 positive cells could not be determined in the first fraction obtained at a flow rate of 4.7 ml/minute as this fraction contained too few cells for analysis. The percentage of positive cells was highest (40%) in the next fraction obtained at a flow rate of 9.0 ml/minute. The value fell markedly to 12% in the next fraction and was $<1\%$ in all subsequent fractions. These data, indicating that lymphoid progenitors are contained predominantly within early fractions, are consistent with MGG differential data which indicated that cells with the morphological appearance of lymphocytes were also predominantly contained within the early elutriation fractions. They also indicate that lymphoid progenitors were not present to a significant degree in fractions 3 and 4, which were those cells selected for further study of changes in inositol metabolism during myeloid differentiation.

In addition to MGG analysis, the cells obtained by pooling elutriation fractions numbers 3 and 4 were also examined as to their degree of granulocytic and monocytic differentiation as assessed by positive staining with the AGF4.48 antibody and for monocyte specific esterase. These results are shown in Table 21. The means of values obtained for positive staining for alpha-naphthyl acetate esterase (12.2%) and with AGF4.48 antibody (1.5%) correlate well with values obtained for

FIGURE 21. PRESENCE OF CD 10 AND CD 34 POSITIVE CELLS WITHIN EACH ELUTRIATION FRACTION



The percentage of antigen positive cells was determined by indirect immunofluorescence. CD 10 values are a single data set and CD 34 data are the mean of values from two similar experiments.

monocytic cells (11.5%) and granulocytic cells (1.7%) as observed on MGG stained preparations. They indicate that the degree of myeloid differentiation was limited in this fractionated population of cells.

Table 21. Characterisation of cells obtained within pooled elutriation fractions Nos. 3 and 4.

Characteristic [1]	% Cells positive Mean	(±SEM)
MGG differential (%)		
Normoblasts	0	
Lymphocytes	14.8	(4.9)
Monocytes	0.1	
Others	2.3	
Blasts	69.8	(4.2)
Promyelocytes	0.6	
Myelocytes	0.4	
Metamyelocytes	0.6	
Segmented/band forms	0.1	
Promonocytes	11.4	(1.2)
ANAE	12.2	(2.0)
AGF 4.48	1.7	(0.6)

1. Data from 5 experiments; AGF 4.48 values from 3 experiments.

CHAPTER 13

THE EFFECT OF TREATMENT WITH PHORBOL MYRISTATE ACETATE (PMA) ON THE DIFFERENTIATION OF BLAST CELLS PURIFIED FROM HUMAN FETAL LIVER.

Previous studies describing the growth and differentiation in liquid suspension culture of mononuclear cells isolated from human fetal liver (Toksoz and Brown, 1984) showed that these cultures generated large numbers of mature neutrophils and macrophages for a period of up to 1 month. This observation indicated that the initial cell population contained cells at early stages of myeloid differentiation. Therefore, similar studies of the extent of myeloid differentiation in liquid suspension culture were carried out on undifferentiated blast cells purified from human fetal liver following erythroblast and macrophage depletion. Cells purified according to the protocol incorporating mechanical disruption of tissue were used for these studies. The effect of phorbol myristate acetate (PMA) on the differentiation of these normal myeloid blast cells was also studied in these experiments. PMA is known to induce rapid monocyte differentiation of the human promyeloid cell line HL60 (Rovera et al, 1979) but its effects on normal progenitor cells had not been studied previously. In these cultures, monocyte differentiation was assessed by enumerating a) cells exhibiting monocytic (promonocytes and monocytes/macrophages) morphology following staining with MGG and b) cells expressing α-naphthyl acetate (monocyte specific) esterase. Granulocyte differentiation was assessed by a) cells exhibiting granulocytic (promyelocyte, myelocyte, metamyelocyte and mature granulocyte) morphology following staining with MGG; b) cells expressing lactoferrin, a known constituent of neutrophil secondary granules and c) cells staining with the AGF4.48 antibody, a neutrophil specific antibody of the CD15 cluster, which identifies cells at or beyond the promyelocyte stage of differentiation.

13.1 Induction of monocyte differentiation by PMA.

PMA was added immediately after establishing cultures of purified blast cells at 2.5 x 10^5/ml. Sixteen hours after the addition of PMA, the morphological appearance of cells in the treated cultures was already

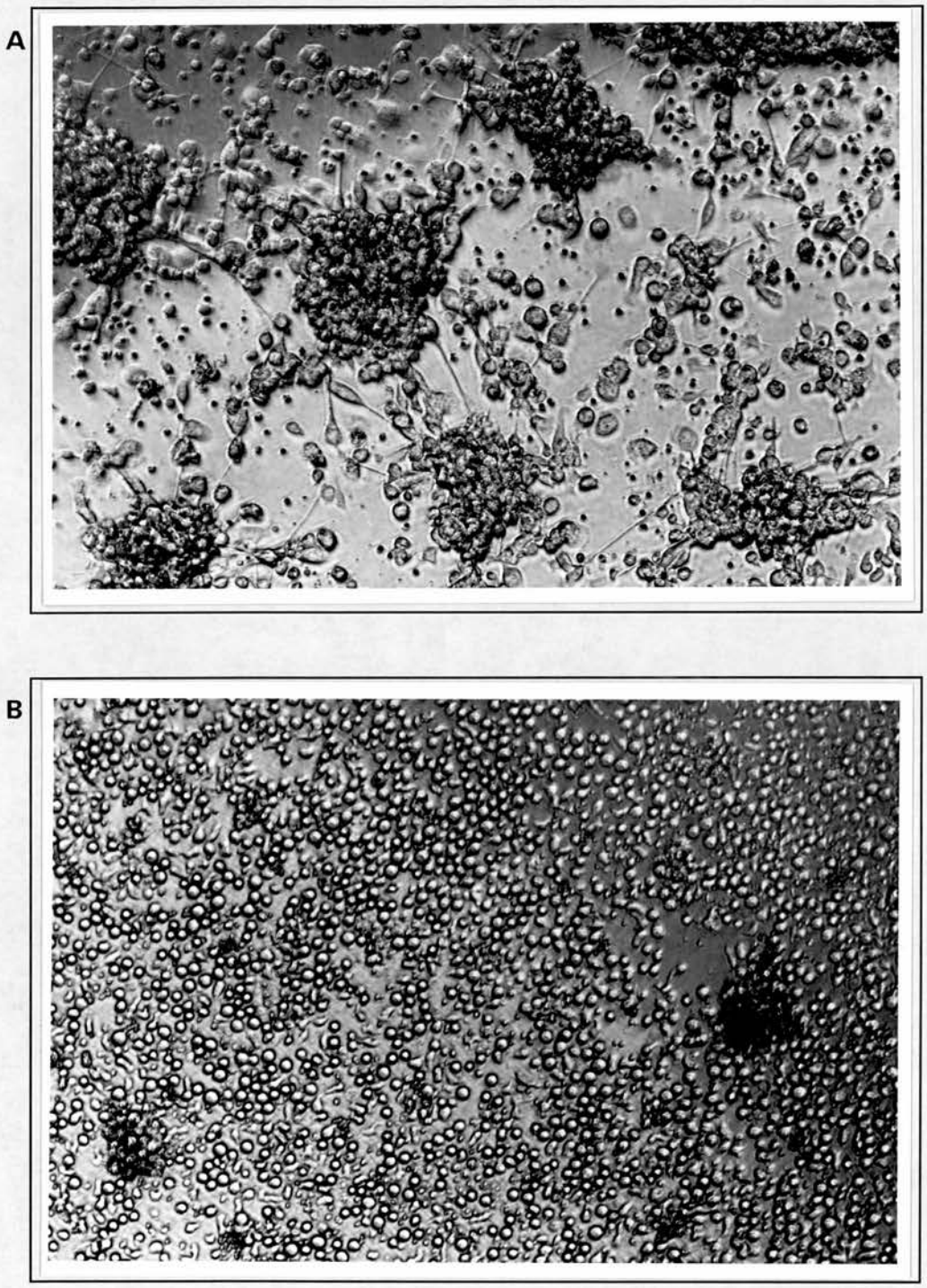
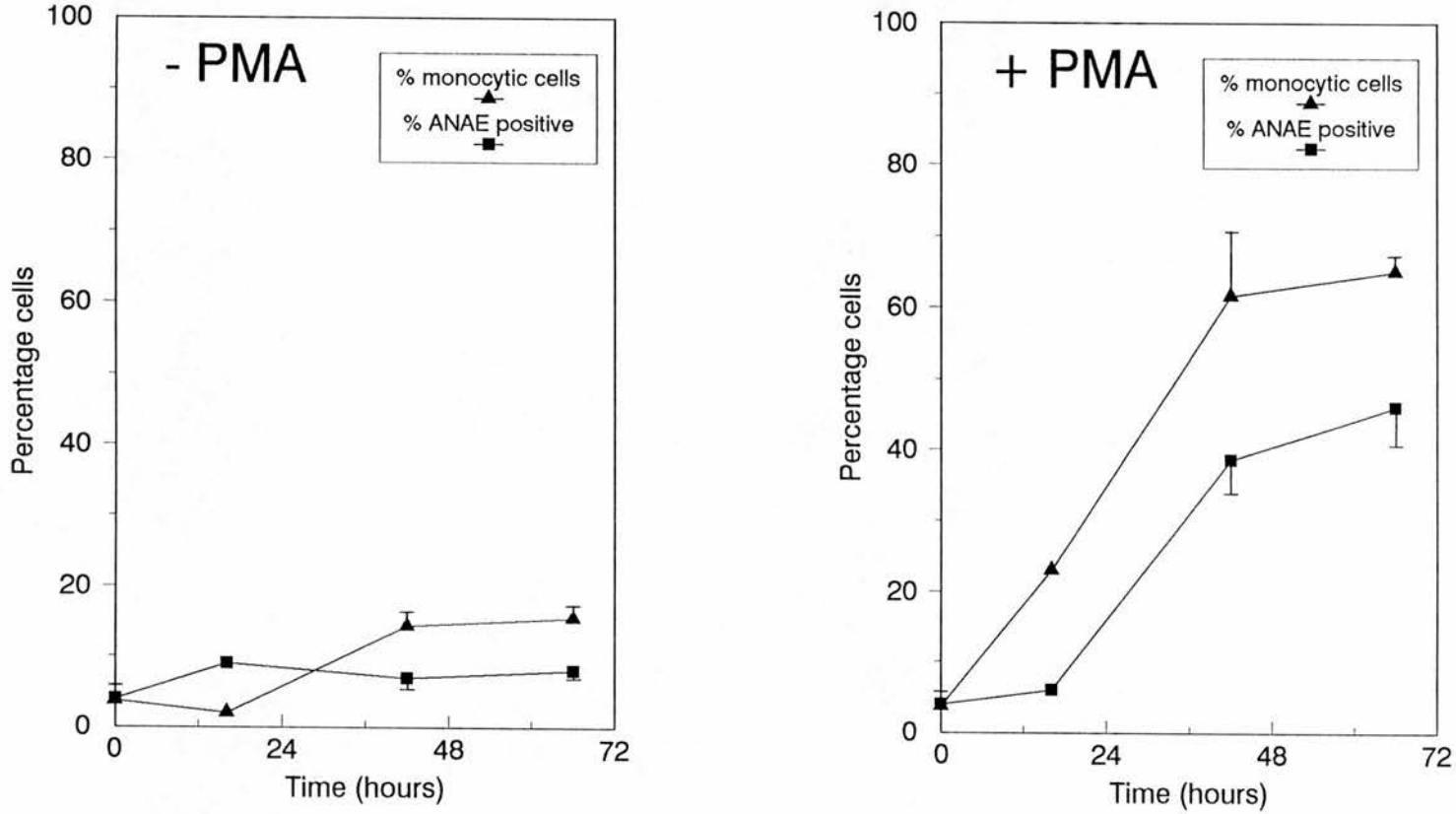


Plate 6. Photomicrographs of undifferentiated blast cells cultured in medium containing fetal calf serum with (6a) and without (6b) the addition of 10nM PMA. Cells in PMA-treated cultures became clumped, adherent, spread and had produced pseudopodia - changes consistent with the cells having undergone monocyte differentiation (x 140).

different from that in control cultures (Plate 6). A considerable proportion of the cells in the PMA-treated wells had become clumped, adherent, spread and had produced pseudopodia. Cells with these features which are consistent with cells having undergone macrophage differentiation were less evident in the control cultures. Monocyte differentiation in PMA-treated cultures was confirmed following assessment of monocytic cells seen in MGG stained cytocentrifuge preparations. Promonocytes, which were more evident in PMA treated cultures, had distinguishing characteristics of increased size with increased quantities of cytoplasm which was less basophilic, often vacuolated, and had irregular edges. Phagocytosis of a few contaminating sheep erythrocytes, from the rosette procedure, was also occasionally seen. The nuclear to cytoplasmic ratios was reduced in these cells and the nucleus was eccentrically placed, reniform in shape and contained nucleoli. More mature cells showed typical macrophage morphology (Plate 7). At time zero, cells with these features accounted for 4% of cells. The addition of 10 nM PMA resulted in an increase of monocytic cells to values of 62% and 65% at 42h and 66h respectively, compared to values of 14% and 16% in control cultures (Figure 22), The rapid morphological differentiation towards macrophages seen with PMA was accompanied by an increase in the number of cells expressing α-naphthyl acetate esterase. Four percent of the initial cell population expressed this enzyme but by 66h, 46% of cells in PMA-treated cultures were positive compared with 8% of control cells (Figure 22; Plate 7).

When expressed in absolute numbers the number of cells expressing α-naphthyl acetate esterase had increased from 1.0×10^4 cells initially to 9.7×10^4 in PMA-treated cultures at 66h compared to 1.8×10^4 in control cultures at this time point. The number of phagocytic cells increased from $<1\%$ to 17% at 66h in PMA-treated cultures as opposed to 6% in control cultures. In both the PMA-treated and control cultures there was no increase in the total number of cells over the 66 hr during which analyses were undertaken and the values obtained for the number of viable cells recovered (80-92%) and cell viability (87-98%) were similar for the treated and untreated cultures throughout the course of each experiment.

FIGURE 22. INDUCTION OF MONOCYTE DIFFERENTIATION WITHIN PURIFIED BLAST CELLS BY PMA



results are means(±SEM) of values obtained from 4 separate experiments

Assessment of cell proliferation by analysis of DNA synthesis was also performed. In control cultures there was a moderate increase in [^{3}H] TdR uptake. Values for dpm incorporated per 10^5 cells were 3.0×10^4 at 12h rising to 5.1×10^4 and 4.2×10^4 at 60 and 84h respectively. PMA treated cultures had proliferated to a lesser extent as shown by [^{3}H] TdR incorporation values of 1.7×10^4, 4.2×10^4 and 3.4×10^4 at 12h, 60h and 84h respectively. Thus it was unlikely that PMA had induced rapid and extensive proliferation of a minor population of the purified blast cells and that this together with loss of a considerable number of cells within another cell population had maintained cell numbers in the PMA-treated cultures. Furthermore, large numbers of dead cells were clearly not present in the wells. Plate 7 illustrates that the frequency of monocytic cells was considerably higher in PMA-treated cultures than in cultures of control cells.

Plate 7(opposite). PMA-induction of monocyte differentiation and inhibition of granulocyte differentiation of undifferentiated blast cells purified from human fetal liver.

Illustrations are photocpoies of colour photomicrographs. Figures a -c are Romanowsky stained preparations; figures d - f are preparations stained for α-naphthyl acetate esterase activity (monocytic cells); and figures g - i are preparations stained by indirect immunofluorescence with AGF4.48 antibody (granulocytic cells) (magnification x 500). Figures a,d,g show cells at 0h; figures b,e,h show control cells at 66h and figures c,f,i show cells treated with PMA for 66h.

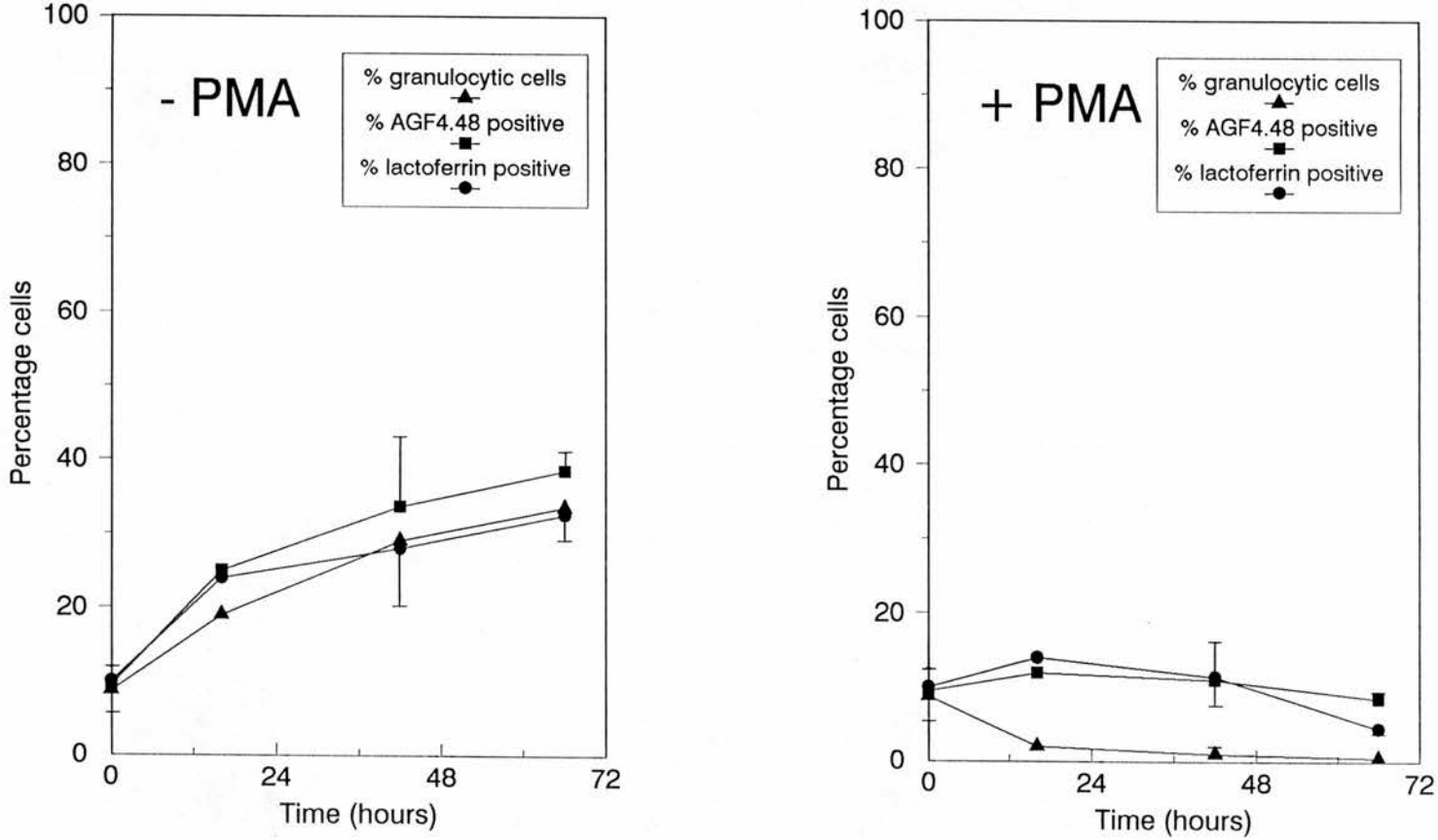
a
b
c
d
e
f
g
h
i

13.2 Inhibition of granulocyte differentiation by PMA.

In PMA-treated cultures the maximum percentage of cells which could be induced to differentiate towards monocytes was 63-67%. At 66h, 31-33% of the remaining cells, as observed in MGG stained preparations had retained a blast cell appearance and only 1% of total cells had differentiated towards neutrophils. In contrast, analysis of MGG stained preparations from control cells showed that, by 66h, 34% of cells exhibited morphological evidence of granulocytic differentiation (Figure 23, Plate 7). To confirm that the addition of PMA had interfered with granulopoiesis within the purified blast cell population the percentage of cells expressing lactoferrin and the neutrophil specific cell surface antigen AGF4.48, which is expressed at the promyelocyte stage of differentiation, were enumerated throughout the time course of PMA-treated and control cultures. Within the starting cell population, 10% of cells contained lactoferrin and expressed the AGF4.48 antigen. By 66h the numbers of lactoferrin and AGF4.48 antigen positive cells in control cultures had increased to 33% and 39% respectively. In contrast, throughout the time course of PMA-treated cultures, the percentage of lactoferrin positive cells remained at the level of 5-14% and those stained by the monoclonal antibody AGF4.48 at 9-12%. At 66h, the mean value for the percentage of AGF4.48 positive cells represented a level of 1.9×10^4 mature granulocytic cells in PMA treated cultures as compared with 9.0×10^4 in control cultures. Initial levels of mature granulocytic cells in the purified blast cell population were in the range of $1.8 - 2.1 \times 10^4$. Plate 7 illustrates that the frequency of mature granulocytic cells was considerably lower in PMA-treated cultures than in cultures of control cells.

To exclude the possibility that the inhibition of granulopoiesis or the promotion of monopoiesis observed was due to indirect effects whereby PMA had induced the small percentage of contaminating lymphocytes to release inhibitory or inductive factors, the following experiment was undertaken. The monoclonal antibody AGF43, which stains both T and B lymphocytes (Fisher et al, 1983), was added to the antibody mixture used to prepare indirect rosettes and this resulted in effective depletion of lymphocytes. In this experiment, light density

FIGURE 23. INHIBITION OF GRANULOCYTE DIFFERENTIATION WITHIN PURIFIED BLAST CELLS BY PMA



results are means(±SEM) of values obtained from 4 separate experiments.

cells prepared from fetal liver following ficoll separation consisted of 56% blasts, 22% normoblasts, 4% macrophages and 19% of cells with the morphological appearance of lymphocytes. Cells subsequently depleted with the Ret40F and 61D3 antibodies were comprised of 77% blasts and 21% of cells with the morphological appearance of lymphocytes. The fetal liver cell population which had been depleted using the three antibodies (Ret40F, 61D3, and AGF43) contained 86% blast cells and only 1% lymphocytes. Parallel studies of the effect of PMA on the above two purified cell populations showed that removal of the contaminating lymphocytes did not affect the rapid induction of monocyte differentiation by PMA or the inhibition of granulopoiesis. At 61h after PMA treatment, cultures established from fetal liver cells depleted by lymphocytes and from those cells contaminated by lymphocytes contained similar numbers of cells expressing α-naphthyl acetate esterase (27% and 21% respectively). In both cultures after treatment with PMA for 61h there was no evidence of neutrophil differentiation as assessed by the percentages of AGF4.48 antigen positive cells ($<1\%$) and cells at the promyelocyte to neutrophil stages of maturation ($<1\%$).

13.3 The effect of treatment with PMA on the expression of cytochemical markers.

The effect of PMA treatment of undifferentiated blast cells on the expression of other cytochemical markers was also studied. The cytochemical stains employed were Sudan Black B, chloroacetate esterase, butyrate esterase and periodic acid-Schiff reagent. Two experiments were carried out where the percentage of cells expressing each marker was assessed initially and after 66 hours in culture. Both experiments gave similar results which are expressed as a mean of the obtained values. To validate the experiments, control data for cell number, viability, results of MGG staining and staining with the AGF4.48 antibody were also assessed in parallel. Analyses for these control data gave results similar to the previous PMA induction experiments. The percentage of cells staining for chloroacetate esterase, a marker for mature granulocytic cells showed good agreement with the percentage of granulocytic cells observed following MGG staining and

the percentage of cells staining with the AGF4.48 antibody in both control and PMA treated cultures (Figure 24). Control cells after 66 hours in culture showed significantly increased numbers of chloroacetate esterase positive cells (12% - 35%) whereas in PMA treated cultures, chloroacetate esterase positive cells fell from 12% to 9%. The percentage of cells staining for butyrate esterase, a monocyte specific esterase, also showed good agreement with data obtained from the previous PMA induction experiments. The percentage of butyrate esterase positive cells increased slightly in control cultures (2% to 7%) but showed a much larger increase in PMA treated cultures (2% to 26%) (Figure 24). These data on the expression of chloroacetate esterase and butyrate esterase support the previous observations on the effect of PMA on the differentiation of myeloid blast cells purified from fetal liver. The percentage of cells staining with Sudan Black B and periodic acid-Schiff reagent showed large increases in both control and PMA treated populations. Sudan Black B positive cells increased from 17% to 88% in control cultures and from 17% to 87% in PMA treated cultures. PAS positive cells increased from 15% to 39% in control cultures and from 15% to 56% in PMA treated cultures. These data for Sudan Black B and PAS staining are consistent with previous observations since these markers are absent in undifferentiated blast cells but present in cells showing evidence of either granulocytic or monocytic differentiation. Their levels were low in the initial undifferentiated blast cell population and increased after 66 hours in culture, over which time period significant differentiation had occurred.

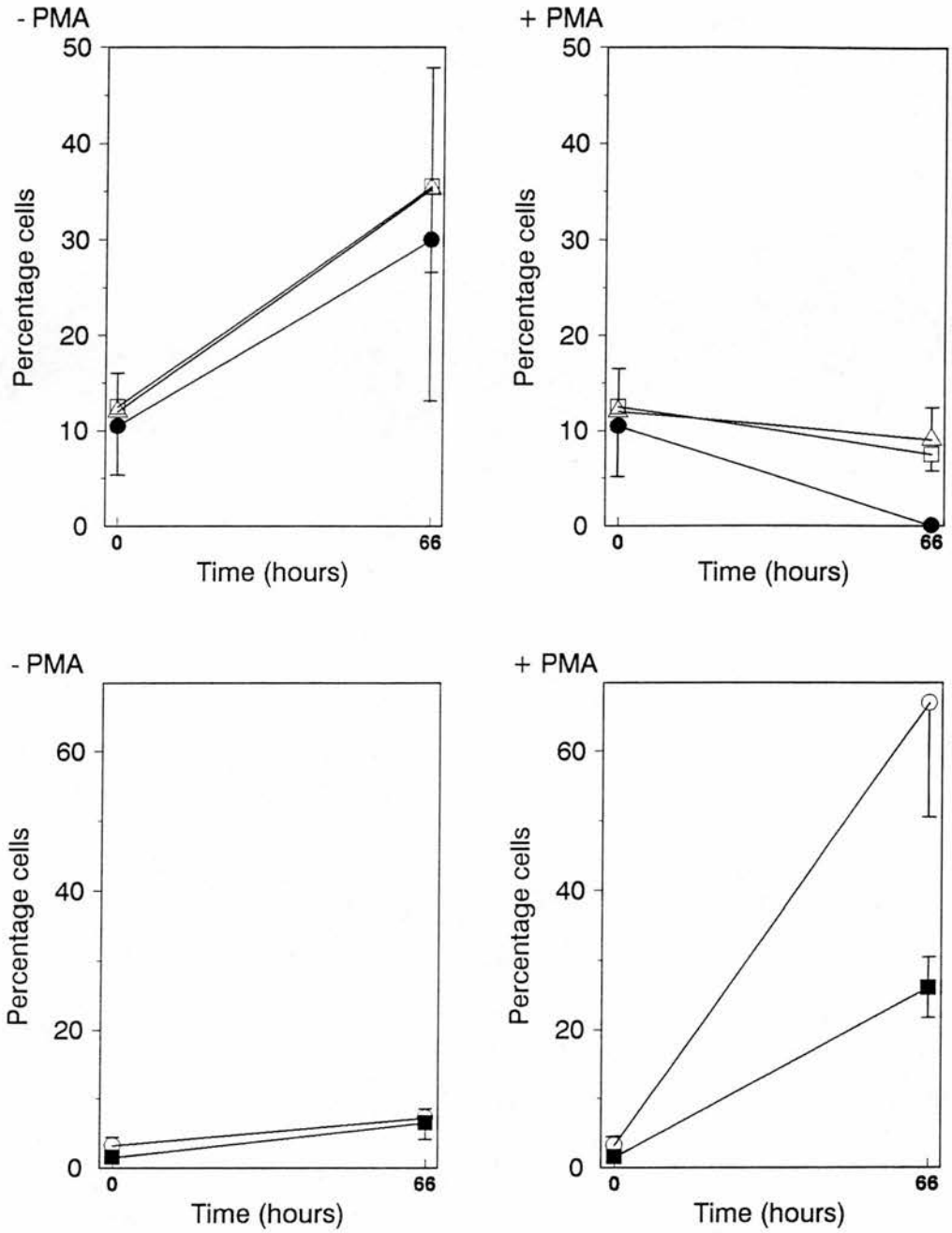


Figure 24. Effect of PMA treatment on the expression of chloroacetate and butyrate esterases within purified blast cells. The percentages of granulocytic cells (●), AGF4.48 positive cells (□), chloroacetate positive cells (△), monocytic cells (○), and butyrate esterase positive cells (■) were determined. The results are means of values (±SEM) from two experiments.

13.4. The effect of treatment of purified blast cells with PMA on the expression of leucocyte surface antigens.

The effect of PMA treatment of undifferentiated blast cells on the expression of leucocyte surface antigens was also studied. The cell surface antigens studied and the results are shown in Table 22. Two experiments were performed where the percentage of cells expressing each marker was assessed initially and after 66h in culture. Both experiments gave similar results which are expressed as a mean of the values obtained. To validate the experiments control data for cell number and viability, and results for MGG staining and staining with the AGF4.48 antibody were also assessed in parallel. Analyses of these control data gave identical results to the previous PMA induction experiments.

The percentage of glycophorin positive cells remained low in all cell populations (<3%) indicating a low initial presence of these cells and no increase in their numbers during culture.

The percentage of cells expressing CD15 on their surface, as detected by the antibody, was higher in control cultures (31%) than PMA treated cultures (1%) and paralleled the results obtained following staining with the AGF4.48 antibody, another antibody of the CD15 cluster (Figure 25). These data are consistent with previous observations that certain CD15 monoclonal antibodies recognise cells of the granulocytic lineage and therefore support the previous observations of the effect of PMA on the differentiation of myeloid blast cells purified from fetal liver.

The CD11c antigen (p150,95) is a member of the CD11 family of cell adhesion molecules which has a unique α-chain sub unit (CD11c). This sub unit combines with a ß-chain sub unit (CD18) which is common to other members of the CD11 family. CD11c has been shown to have C3b receptor (CR4) activity (Myones et al, 1988) and although it is weakly expressed in neutrophils and cells from cases of certain chronic B-cell leukaemias, its expression is strongly linked to cells of the monocytic lineage. CD11c is strongly expressed on monocytes and monocyte derived macrophages (Uchiechowski and Schmidt, 1989;

Table 22. Effect of PMA treatment of undifferentiated myeloid blast cells purified from human fetal liver on the expression of leucocyte surface antigens.

	% of population positive (±SEM)		
Surface Antigen	Undifferentiated Blasts Time 0h	Blast cells (- PMA)[1] 66h	Blast cells (+ PMA)[1] 66h
Erythroid			
Glycophorin C	1 (0.4)	0 (0)	2.5 (2.5)
Myeloid			
CD11c	7 (0.8)	11 (0.5)	67 (10.5)
CD15	8 (1.0)	31 (1)	7 (1)
CD14	3 (1.3)	19 (1.5)	18 (4)
Lymphoid			
CD3 (T cell)	1 (0.6)	3 (3)	<1 (0.5)
CD5 (T cell, B cell subset)	4 (1.5)	1 (1)	2 (1)
CD19 (B cell)	14 (7)	3 (0)	15 (0.5)
CD10 (B cell progenitor)	18 (3.3)	8 (3.5)	4 (0)
Negative control[2]	2 (0.8)	5 (2)	2.5 (1.5)

1. results are mean values (±SEM) from 2 experiments
2. cells were stained using a monoclonal antibody to rotavirus.

Myones et al, 1988). Expression of CD11c is increased on monocytes treated with PMA (Mentizer et al, 1988) and is also increased on HL60 cells and U937 cells induced to differentiate along the monocytic pathway by PMA (Dudley et al, 1989; Pedrinaci et al, 1989). Other studies of acute myeloid leukaemia cells have shown a strong correlation with CD11c expression and cells exhibiting evidence of monocytic maturation (Master et al, 1989; Scott et al, 1990 and Tucker et al, 1990). In these experiments the percentage of cells expressing CD11c showed a significant increase in PMA-treated cultures of blast cells from 7% to 67% whereas in control cultures there was little change (7% to 11%) (Figure 4).

The CD14 antigen is reported to be associated with monocytes. The percentage of cells expressing CD14, as detected by the antibody, showed an equivalent moderate increase from 3% to 18% and to 19% in control cultures. The percentages of mature T cells, which expressed the CD3 and CD5 antigens were low in both control and PMA treated cultures ($<4\%$), indicating that mature T cells did not develop in these culture conditions.

Cells which expressed the CD10 antigen, which is present on B cell progenitors and mature granulocytes, decreased from 18% in the initial blast cell population to 8% in control populations at 66h and to 4% in PMA-treated cultures. Percentages of cells which expressed the CD19 antigen fell from 14% to 3% in control cultures but showed no change in PMA-treated cultures (15%). Therefore there was no evidence to support the growth and differentiation of lymphoid progenitor cells within the cultures of purified blast cells.

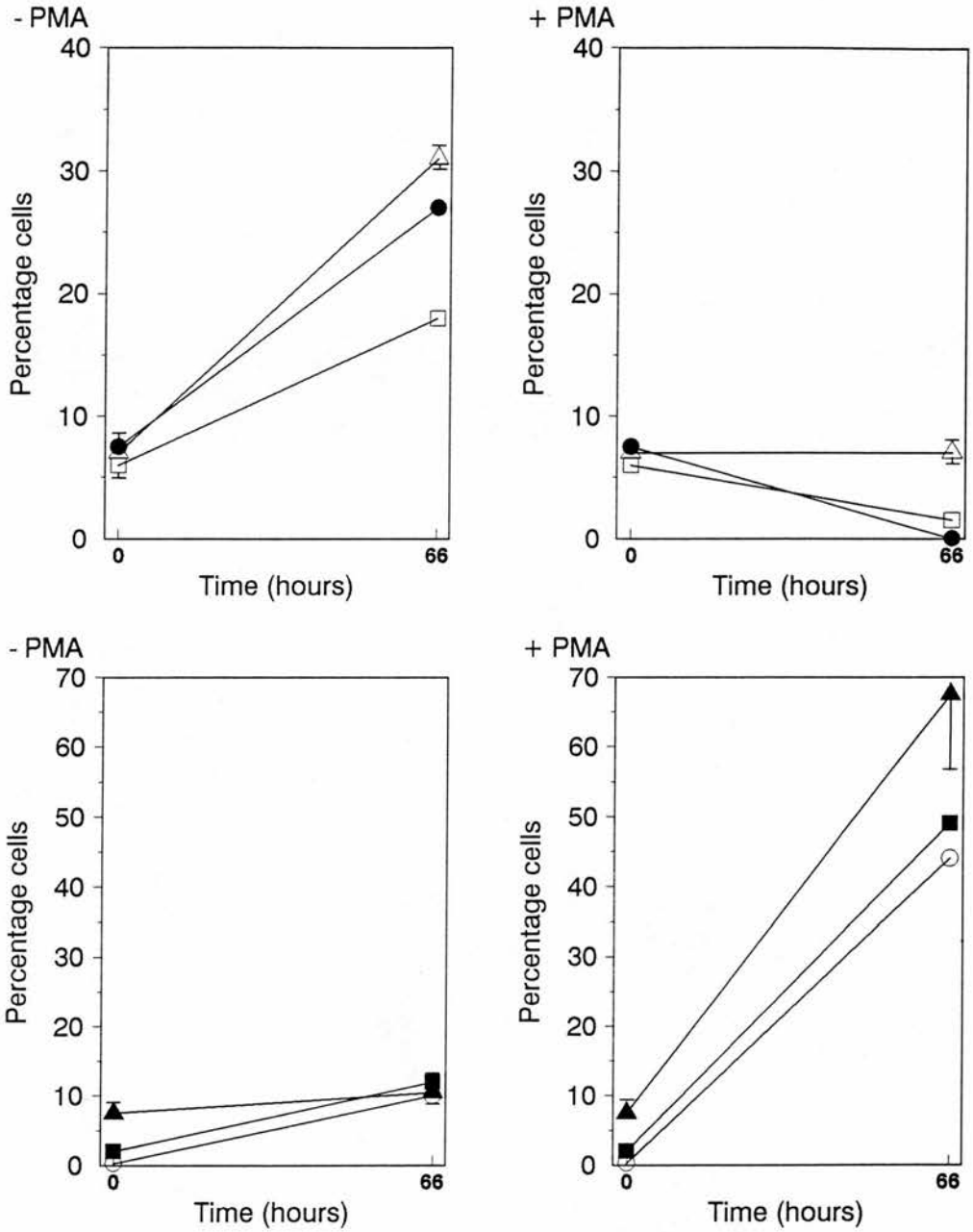


Figure 25. Effect of PMA treatment on the expression of CD 15 and CD 11c antigens by purified blast cells. The percentages of granulocytic cells (●), AGF.4.48 positive cells (□), CD 15 positive cells (△), monocytic cells (○), ANAE positive cells (■), and CD 11c positive cells (▲) were determined. The results are the means of values from two experiments. SEM are shown for CD 15 and CD 11c values.

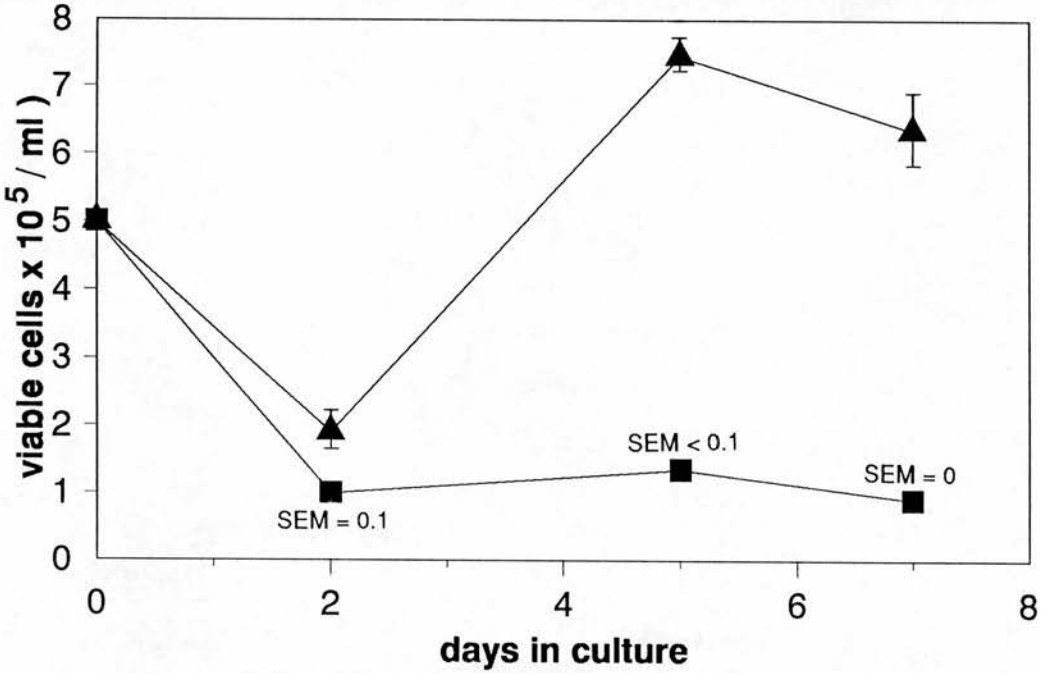
CHAPTER 14.
CULTURE OF FETAL LIVER CELLS UNDER SERUM-FREE CONDITIONS

Studies, outlined in the previous chapters, on cells purified from human fetal liver following erythroblast and macrophage depletions indicated that the population of cells consisted mainly of undifferentiated myeloid blast cells which were capable of either granulocytic or monocytic differentiation. In this manner, the normal cell population resembled the human cell line HL60, which is an undifferentiated myeloid cell line capable of either granulocyte or monocyte differentiation. Previous studies within the laboratory had delineated intracellular metabolic events within HL60 cells which occur during either PMA-induced monocyte differentiation or DMSO-induced granulocyte differentiation of these cells. These studies included investigation of protein phosphorylation and of inositol metabolism within HL60 cells (French et al, 1991). It was, therefore, of interest to determine whether the events described in studies of HL60 cells occur within the normal population of undifferentiated myeloid blast cells undergoing differentiation. To permit studies of intracellular inositol metabolism in normal myeloid blast cells it was necessary to maintain and grow the blast cells in culture so as to allow incorporation of [^{3}H]-inositol. It was also necessary to maintain the cells in serum-free conditions so as to minimise the usage of radiolabelled inositol. Furthermore, the studies of inositol metabolism within HL60 cells were undertaken using cells grown under serum-free conditions and in medium containing a low amount of inositol. Therefore, the aim was to maintain the blast cells in the serum-free and low inositol medium used in the HL60 studies. Once equilibrium labelling of inositol metabolites had been achieved within blast cells, the changes in metabolism which occur when the blast cells are induced to differentiate by PMA could be investigated.

14.1 Culture of normal myeloid blast cells, purified following enzyme digestion of liver tissue and erythroblast and macrophage depletion in serum-free medium.

Preliminary experiments to determine the survival of undifferentiated blast cells under serum-free conditions were carried out on blast cells

FIGURE 26. MAINTENANCE OF NORMAL MYELOID BLAST CELLS IN SERUM-FREE MEDIUM CONTAINING IL-3.

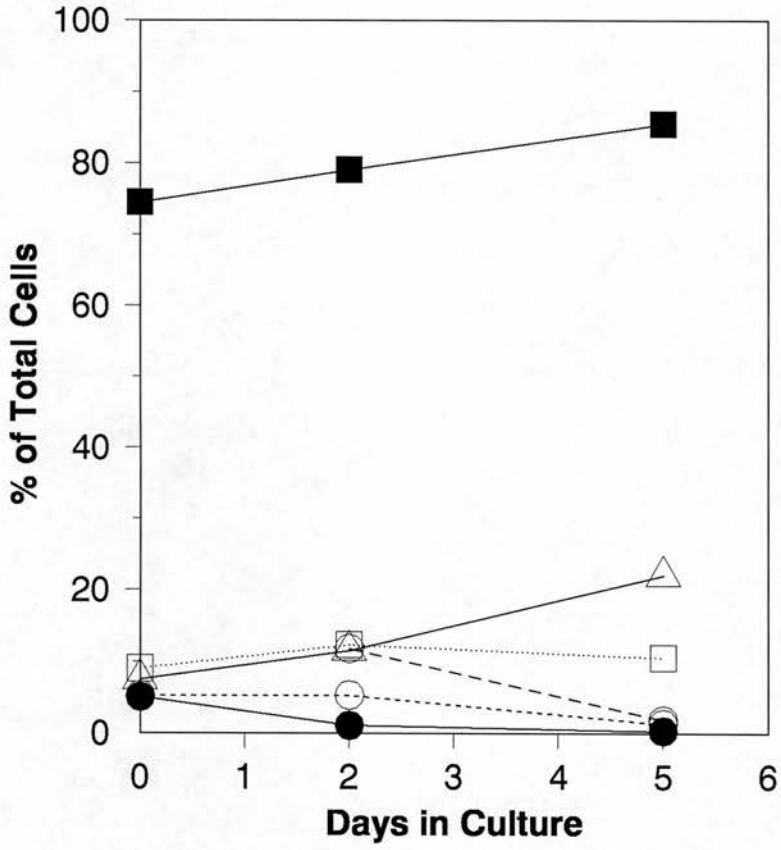


Cells purified from human fetal liver following erythroblast and macrophage depletion were cultured in serum-free medium containing 1mg/L inositol with (▲) and without (■) the addition of 100u/ml IL-3. Data are means of values (±SEM) from two experiments.

purified following enzyme digestion of liver tissue and erythroblast and macrophage depletion. In order to have adequate numbers of blast cells, with which to carry out studies of the survival of cells under serum-free conditions, the blast cells were not further purified by counter current elutriation. Figure 26 shows the survival of undifferentiated blast cells when cultured under serum-free conditions in medium containing low amounts of inositol with and without the addition of a high concentration of IL-3 (100U/ml). The results are means of the values obtained (+SEM) from two experiments. Blast cells cultured in serum-free medium in the absence of IL-3 died rapidly and surviving cells did not proliferate after a further culture period. In contrast, blast cells exhibited much better survival in serum-free medium containing IL-3. Although all numbers fell off within the first two days in culture, after this time period cells showed evidence of proliferation. In cultures containing IL-3 on day +2 the number of cells in culture was 1.9 x 10^5/ml, whereas, on day +5 and day +7 the average of the values obtained for the number of cells in culture was 7.45 and 6.35 x 10^5/ml respectively. The mean of the values obtained for cell viability on day +5 and day +7 was 90% and 85% respectively.

Equilibrium labelling of inositol metabolites with [^{3}H]-inositol requires the cells to be grown for five days in culture and maintained in an undifferentiated state. During the initial five day period of growth of blast cells in the presence of IL-3, the cells did not differentiate to a large extent (Figure 27). Cells classified as blasts or promyelocytes remained high throughout the 5 days of culture. At time 0, these cells accounted for 75% of cells and by day +5 they accounted for 85% of cells. These cells were nearly all undifferentiated blast cells, until day +5 when a significant number of cells containing azurophil granules (31%) were observed which were classified as promyelocytes. However, these cells had not shown marked evidence of granulocytic differentiation as indicated by the extremely low percentage of cells staining with the AGF4.48 antibody (2%). The extent of either granulocyte or monocyte differentiation within the five day culture period was limited as indicated by the following observations. The percentage of cells classified as mature granulocytes at or beyond the myelocyte stage of differentiation accounted for 5% of the starting

FIGURE 27. MAINTENANCE OF UNDIFFERENTIATED BLAST CELLS IN SERUM-FREE MEDIUM CONTAINING IL-3.



Cells purified from human fetal liver following erythroblast and macrophage depletion were cultured in serum-free medium containing 1mg/l inositol and 100U/ml IL-3. Differentiation was assessed by the percentage of α-naphthyl acetate esterase positive cells(Δ), AGF4.48 positive cells(—○—), and the identification of cells following staining with May-Grunwald-Giemsa; blasts/promyelocytes(■), promonocytes/monocytes (□), mature granulocytes (--○--) and lymphocytes(●). The results are means of values obtained from two experiments.

population of cells, and by day +5 accounted for only 2% of cells. At this point the percentage of AGF4.48 positive cells was 2%. The percentage of cells classified as either promonocytes or monocytes accounted for 9% of the starting population of cells and by day +5 accounted for 11% cells. Cells positive for α-naphthyl acetate esterase accounted for 8% of the starting population of cells and by day +5 this value had increased to 22%. The percentage of cells with the morphological appearance of lymphocytes was 5% of the starting population of cells and by day +5 this value was <1%. By day +7 of culture, however, further differentiation had occurred with significant increases in the percentages of promonocytes/monocytes (46%) and cells positive for α-naphthyl acetate esterase (50%).

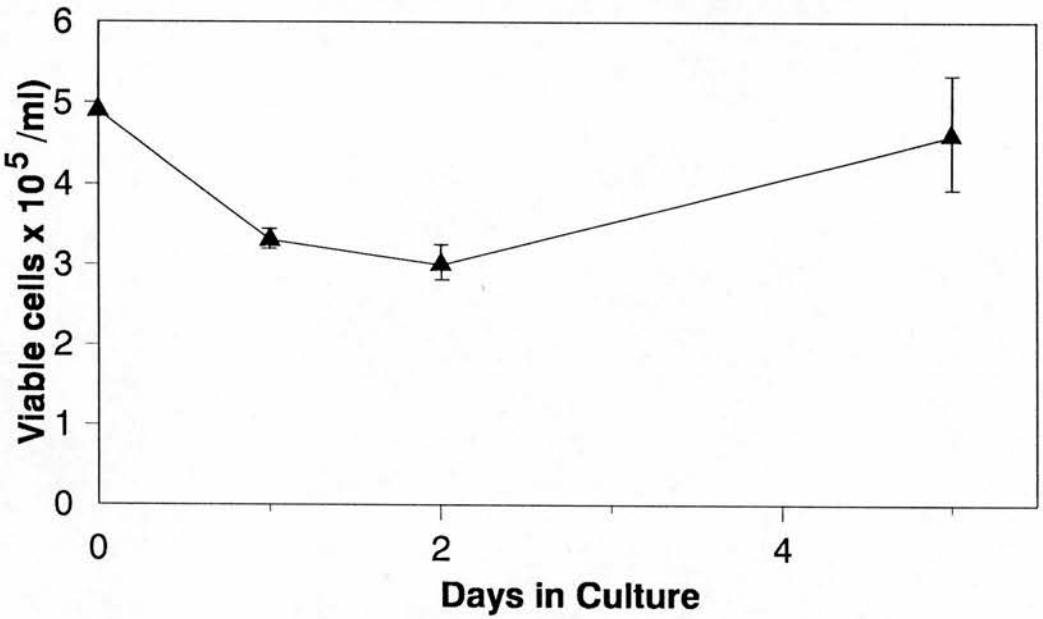
These observations revealed that the presence of IL-3 was necessary for the maintenance and growth of undifferentiated blast cells in culture. The experiments to study inositol metabolism, presented in the following chapter, were undertaken using elutriated blast cells so as to obtain a more homogeneous blast cell population.

14.2 Culture of normal myeloid blast cells purified after cell elutriation in serum-free medium containing IL-3.

For the studies of inositol metabolism within blast cells they were maintained in RPM1 1640 medium containing a low amount of inositol (1 mg/l), serum-free additives and IL-3. It was necessary to determine whether the elutriated blast cells could be maintained in an undifferentiated state under these culture conditions. In 5 experiments cells pooled from CCE fractions 13.3 and 17.8 mls/minute were cultured for 5 days in serum-free medium containing 100 U/ml IL-3. The extent to which the elutriated blast cells were maintained in an undifferentiated form by culture in IL-3 is shown in Figures 28 and 29. The results at time zero and day +5 are means (+SEM) from 5 data points and the intervening points are means (+SEM) from 2 or 3 data points. Figure 28 shows that the elutriated cells were maintained in culture for 5 days in serum-free medium containing a low amount of inositol and IL-3. Up to day +2 of culture cell numbers fell from 4.9 x 10^5/ml to 3.0 x 10^5/ml. However, beyond day +2 there was a recovery in cell numbers to a value of 4.6 x 10^5/ml on day +5. Therefore, there was not extensive growth of the elutriated blast cells in the presence of IL-3. The mean of the values obtained for cell viability was 94% at time zero, 75% at day +2 and was 85% at day +5.

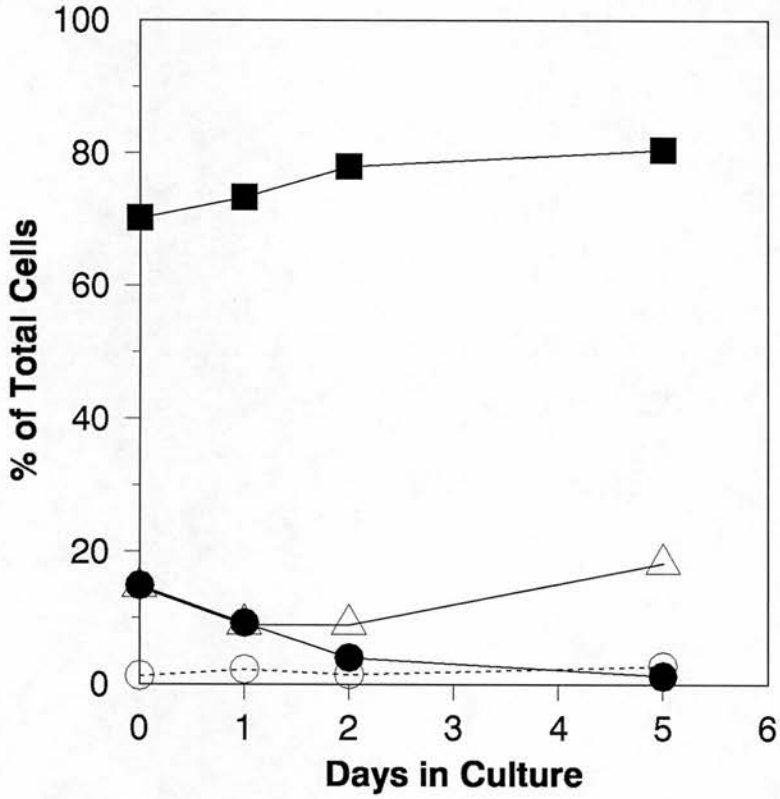
Figure 29 illustrates that the elutriated blast cells were maintained in an undifferentiated state during the same study period. In these cultures the percentage of the initial population classified as blast cells was 70%. By day +5 this value was 68%. Cells containing azurophil granules which were classified as promyelocytes accounted for <1% of cells up to day +2 of culture and on day +5 of culture accounted for 15% cells. However, as in the previous study of non-elutriated cells, these cells had not shown marked evidence of granulocyte differentiation as the percentage of cells positive for the AGF4.48 antibody on day +5 was low (3%). The percentage of cells positive for α-naphthyl acetate esterase was 14% at time zero. By day +5 this value was 18% indicating that monocyte differentiation had not occurred to any significant degree during the 5 day culture period. The percentage of cells with the morphological appearance of lymphocytes, was 15% at

FIGURE 28. MAINTENANCE OF NORMAL MYELOID BLAST CELLS PURIFIED BY ELUTRIATION IN SERUM-FREE MEDIUM CONTAINING IL-3.



Cells obtained from pooled elutriation fractions 3 and 4 were cultured in serum-free medium containing 1mg/L inositol and 100U/ml IL-3. Data are means of values (±SEM) from 5 experiments.

FIGURE 29. MAINTENANCE OF UNDIFFERENTIATED BLAST CELLS PURIFIED BY ELUTRIATION IN SERUM-FREE MEDIUM CONTAINING IL-3.



Cells obtained from pooled elutriation fractions 3 and 4 were cultured in serum-free medium containing 1mg/l inositol and 100U/ml IL-3. Differentiation was assessed by the percentage of α-naphthyl acetate esterase positive cells(Δ), AGF4.48 positive cells(○), and the identification of cells following staining with May-Grunwald-Giemsa; blasts/promyelocytes(■) and lymphocytes(●). The results are means of values obtained from 5 experiments.

time zero. By day +5, however, this value was 1%, indicating that these cells were not maintained in the 5 day culture period. In a single experiment the blast cells were cultured up to 7 days. At day +7 the number of viable cells in culture was 5.44 x 10^7/ml and 82% of cells were viable. The percentage of blast cells was reduced at 47%. There was evidence of monocyte differentiation as indicated by the percentage of cells classified as promonocytes (17%) and positive for α-naphthyl acetate esterase (30%). An increased percentage of cells contained azurophil granules, most of which were classified as promyelocytes, but the percentage of AGF4.48 positive cells remained low (2%). After culturing the blast cells for 7 days, cells with the morphological appearance of lymphocytes were not present. These data showed that the elutriated blast cells could be maintained by culturing the cells in serum- free conditions in medium containing a low amount of inositol (1 mg/L) and a high concentration of IL-3 (100 U/ml). This permitted equilibrium labelling with labelled inositol to be undertaken so as to identify the pattern of inositol metabolites within undifferentiated blast cells. Furthermore, as presented in the next chapter, the changes in inositol metabolism during monocyte induced differentiation of blast cells, as induced by PMA, could then be studied.

CHAPTER 15

THE PATTERN OF INOSITOL METABOLITES WITHIN NORMAL MYELOID BLAST CELLS AND CHANGES DURING THEIR DIFFERENTIATION TOWARDS MONOCYTES.

Inositol, which is a cyclic polyol synthesised from glucose, forms an integral part of molecules which are key regulators of important events within eukaryotic cells. These molecules include membrane bound inositol lipids and intracellular inositol phosphates which are involved in processes such as intracellular signalling pathways which regulate the control of cell proliferation and differentiation (Michell et al, 1990). For example, receptor activation of phosphatidyl inositol 4,5-bisphosphate (Ptd Ins $(4,5)P_2$) hydrolysis by membrane bound phosphoinositidase C generates inositol 1,4,5-triphosphate and 1,2-diacylglycerol. Inositol 1,4,5-triphosphate ($Ins(1,4,5)P_3$) causes the release of calcium from intracellular stores and its inositol phosphate metabolite inositol 1,3,4,5-tetrakisphosphate (Ins $(1,3,4,5)P_4$) controls the influx of calcium into the cell from the external medium. The increase in intracellular calcium concentration results in conformational changes in calcium binding proteins, such as calmodulin, which activate cellular enzymes, especially kinases. Subsequent phosphorylation events induce secondary changes, such as cell proliferation, by mechanisms which are as yet poorly understood. Membrane hydrolysis of Ptd Ins $(4,5)P_2$ also releases 1,2-diacylglycerol which activates protein kinase C, therefore inducing further intracellular phosphorylation events. The phorbol ester, PMA directly activates protein kinase C, and may therefore mimic key regulatory events which are of physiological importance.

Other inositol phosphates include polyphosphorylated forms such as inositol 3,4,5,6-tetrakisphosphate ($Ins(3,4,5,6)P_4$), inositol 1,3,4,5,6-pentakisphosphate ($Ins(1,3,4,5,6)P_5$) and inositol-hexakisphosphate (Ins P_6) which are found in abundance within a number of cell types including HL60 cells. These polyphosphates appear to be formed and metabolised by largely unknown metabolic pathways which do not involve lipids. (Shears, 1989). The studies on HL60 cells performed in the laboratory (French et al, 1991) have delineated the pattern of

inositol metabolites within HL60 cells and have documented changes in these metabolites which occur in association with either DMSO-induced neutrophil differentiation or PMA-induced monocyte differentiation of these cells. It was therefore of interest to study the pattern of inositol metabolites in normal myeloid blast cells and to determine whether change in these metabolites occurred in association with PMA-induced monocyte differentiation of these cells.

15.1 The pattern of inositol metabolites within normal myeloid blast cells.

These studies of intracellular inositol metabolism in normal myeloid blast cells were performed in collaboration with Dr P French (Department of Biochemistry) and Dr C Bunce (Department of Immunology). Undifferentiated myeloid blast cells purified following elutriation were cultured in serum-free medium containing 100 U/ml IL-3, 1 mg/l inositol and 2 μCi/ml [^{3}H]-inositol. After 5 days in culture to permit equilibrium labelling of inositol metabolites with [^{3}H]-inositol, intracellular inositol phosphates were extracted and quantified using high pressure liquid chromatography (HPLC). The separation by HPLC of radiolabelled inositol metabolites of normal human myeloid blast cells is shown in Figure 30a and data obtained from identical studies performed on HL60 cells (kindly provided by Dr French and Dr C Bunce) are presented for comparison (Figure 30b). The traces show the ability of HPLC to clearly separate various labelled inositol compounds and peaks relating to particular inositol phosphates have been identified. The identification of individual peaks within HL60 cells has been studied in detail by a combination of methods involving 1) co-migration of known standard inositol compounds and 2) degradation studies involving a) periodate oxidation, reduction and dephosphorylation of separated fractions followed by HPLC separation of the resulting polyols and b) ammonia hydrolysis of separated fractions followed by HPLC separation of resulting monophosphates. These techniques, however, were unable to distinguish between enantiomeric forms of individual inositol phosphates. The principles and details of these identification methods are described in detail in French, 1990.

Figure 30a. HPLC Separation of the inositol metabolites in Normal Human Myeloid Blast Cells

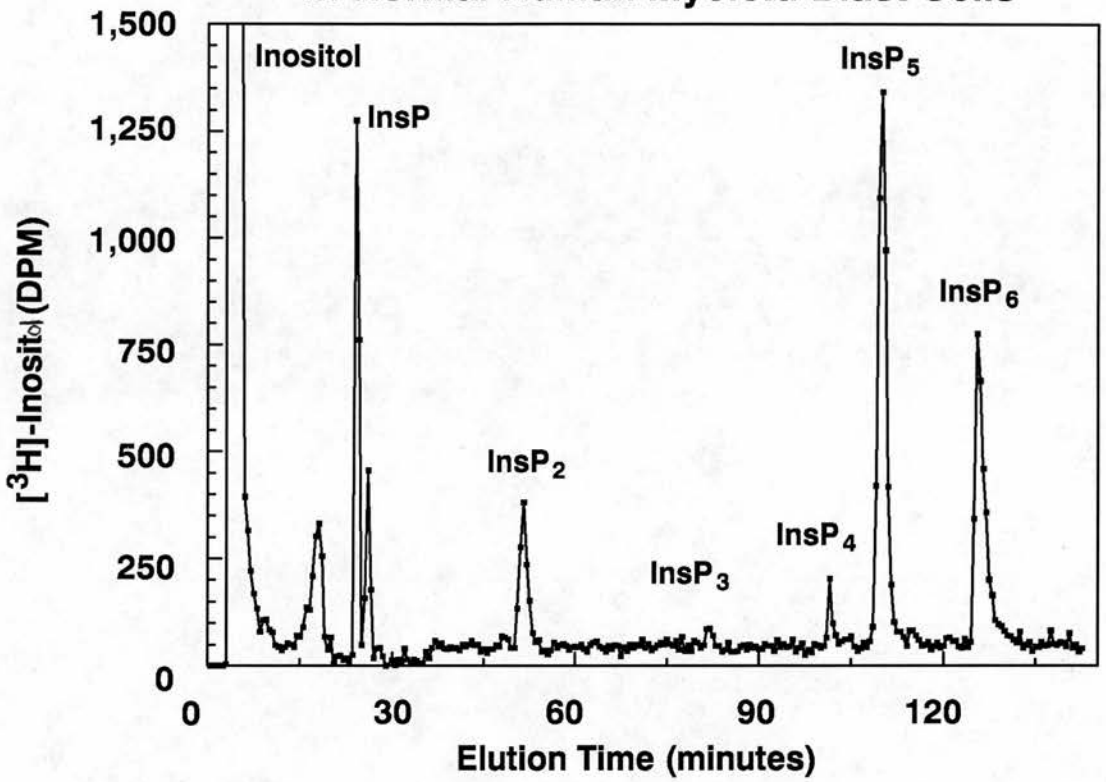
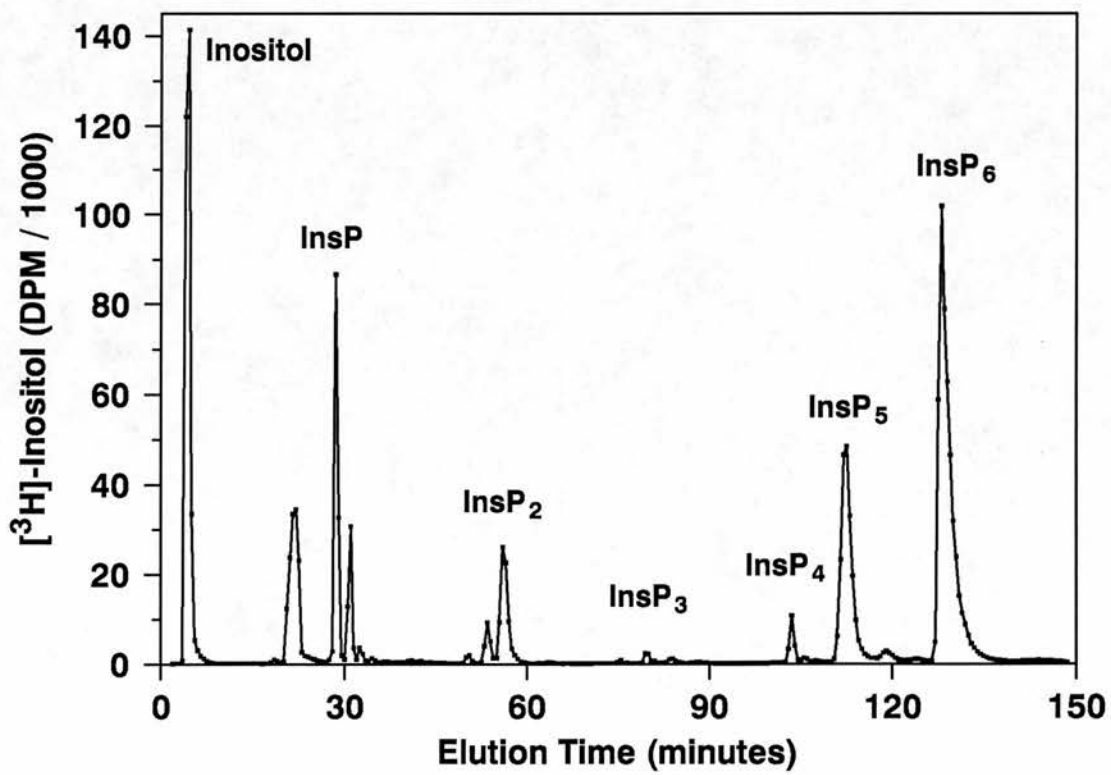


Figure 30b. HPLC Separation of the Inositol metabolites in HL60 cells



The identity of peaks separated within normal myeloid blast cells have been assigned with respect to their co-elution with peaks containing known inositol metabolites from HL60 cells. The first column represents total unchanged myo-inositol and the next fraction represents a weakly charged and as yet unidentified inositol derivative that may be glycerophospho-inositol (GPI). Subsequent labelled peaks represent inositol phosphates, phosphorylated at increasing numbers of sites on the 6 carbon ring of myo-inositol. For example, the peak labelled Ins P for HL 60 cells represents total inositol monophosphate compounds and contains two large and one very small third peak identifying different isomeric forms of inositol monophosphate. The first and largest peak, identified following co-migration with the 14C-Ins (3)P standard, contained Ins (3)P or its enantiomer Ins (1)P. The second smaller peak contained Ins (2)P and the third small peak contained 3 isomers which could not be further separated under the conditions used - Ins (4)P or its enantiomer pair Ins (6)P and Ins (5)P. These inositol monophosphate peaks were similarly represented in normal myeloid blast cells.

The peak labelled Ins P_2 for HL60 cells represents total inositol biphosphate compounds and contained 3 peaks identifying different isomeric forms of inositol biphosphate. The first small peak contains inositol (1,3) biphosphate (Ins (1,3)P_2). The next peak and second most abundant peak in the inositol biphosphate region contained inositol (1,4) biphosphate (Ins(1,4)P_2) or its enantiomeric form inositol (3,6) biphosphate (Ins (3,6)P_2). The third and most abundant peak in the biphosphate region was unidentified. The peak, co-eluting with Ins (1,4)P_2, was the dominant inositol biphosphate peak present in normal myeloid blast cells.

The peak labelled Ins P_3 represents total inositol triphosphate compounds which are present only in small amounts in both HL60 cells and normal myeloid blast cells. Within HL60 cells four separate peaks within the inositol triphosphate region could be identified by HPLC analysis. The first peak contained insufficient material to undertake structural analysis studies and the constituents of the second and most abundant peak could not be definitely identified. The third peak contained inositol (1,2,3) triphosphate (Ins (1,2,3)P_3) and the fourth

peak contained inositol (1,4,5,) triphosphate (Ins(1,4,5)P_3) or inositol (1,4,6) triphosphate (Ins(1,4,6)P_3) and/or their respective enantiomers Ins (3,5,6)P_3 or Ins (3,4,5)P_3 with a small amount of inositol (3,4,5) triphosphate (Ins(3,4,5)P_3) or its enantiomer inositol (1,5,6) triphosphate (Ins(1,5,6)P_3).

The fraction labelled Ins P_4 for HL60 cells represents total inositol-tetrakisphosphate compounds and separates as one large and one very small peak containing different isomeric forms of inositol tetrakisphosphate. The first and by far the most abundant peak of this region contained inositol (1,3,4,5) tetrakisphosphate (Ins(1,3,4,5,)P_4), which accounted for 85% of the peak, and two other isomeric forms, present in much smaller amounts, inositol (3,4,5,6) tetrakisphosphate (Ins(3,4,5,6)P_4) and inositol(1,4,5,6) tetrakisphosphate (Ins(1,4,5,6)P_4). The minor adjacent peak contained inositol (1,2,5,6) tetrakisphosphate (Ins(1,2,5,6)P_4) or its enantiomer inositol (2,3,4,5) tetrakisphosphate (Ins(2,3,4,5)P_4) and either one or a mixture of the following isomers Ins (1,2,4,5)P_4, Ins (1,2,3,5)P_4, Ins (1,2,4,6)P_4 and Ins (2,4,5,6)P_4. A large Ins P_4 peak was similarly present in normal myeloid blast cells, but its contents were not further defined.

The peak labelled Ins P_5 for HL60 cells represented a single isomer of inositol pentakisphosphate, namely inositol (1,3,4,5,6)P_5. This was the most abundant inositol phosphate present in normal myeloid blast cells and was expressed in terms relative to other inositol phosphates at higher levels in normal myeloid blast cells than in HL60 cells. In HL60 cells a small peak between Ins P_5 and Ins P_6 contained a small amount of other Ins P_5 isomers namely either inositol (1,2,4,5,6)P_5 or Ins (2,3,4,5,6)P_5.

The peak labelled Ins P_6 contained the single form of inositol hexakisphosphate which is also known as phytic acid. This compound was present in large amounts in both HL60 cells and in normal myeloid blast cells.

From knowledge of the total number of undifferentiated myeloid blast cells, calculations for mean cell volume, the specific activity of [^{3}H]-

inositol in the culture medium and the total radioactivity present in each peak separated by HPLC, the total intracellular concentrations of inositol metabolites have been calculated (Table 23). These data are means of (+SEM) the values obtained from 10 experiments and represent total intracellular concentrations. No allowance could be made for possible compartmentalisation of inositol metabolites within the cell, under which circumstances local concentrations of inositol metabolites would be increased.

Table 23. Intracellular concentrations of inositol and inositol metabolites in normal myeloid blast cells.

Inositol metabolite[1]	Concentration (μM) mean (±SEM) [2]	
Inositol	2610	(1200)
glycerophospho-inositol (GPI)	22.4	(2.1)
Ins P	16.2	(2.1)
Ins P2	9.4	(4.6)
Ins P3	0.8	
Ins P4	4.2	(0.7)
Ins P5	37.2	(2.7)
Ins P6	30.5	(5.1)

1,2. Cells were cultured for six days in serum-free medium containing 100 U/ml IL-3 and [^{3}H]-inositol. Data are means (+SEM) of values obtained from ten experiments except for Ins P_3 which represents one determination only. Inositol phosphates are labelled Ins P to Ins P_6 and values represent total concentration of inositol polyphosphate isomers.

Analysis of the total intracellular concentrations of the metabolites of myo-inositol present in normal myeloid blast cells yielded a complex picture. The free inositol concentration was 2600μM which was significantly higher than the 5.55μM inositol concentration present in the growth medium. These data suggest that inositol is accumulated by active transport within these cells. There were significant levels of a number of inositol metabolites within normal myeloid blast cells, namely the putative glycerophospho-inositol(GPI) peak, the inositol monophosphate peak (InsP), the inositol biphosphate peak ($InsP_2$), the inositol tetrakisphosphate peak ($InsP_4$), the inositol pentakisphosphate peak ($InsP_5$) and the inositol hexakisphosphate peak ($InsP_6$). The combined concentrations of Ins (1,3,4,5,6)P_5 and $InsP_6$ accounted for half of the aqueous soluble inositol within normal myeloid blast cells. The concentrations of Ins (1,3,4,5,6)P_5 and Ins P_6 were 37.2 μM and 30.5 μM respectively. These higher inositol phosphates were also present in high concentrations within HL60 cells. The peak containing the Ins (1,4,5) P_3 isomer could not be clearly identified in the case of the normal blast cells. The relative amounts of inositol metabolites contained within normal myeloid blast cells and within HL60 cells are discussed later.

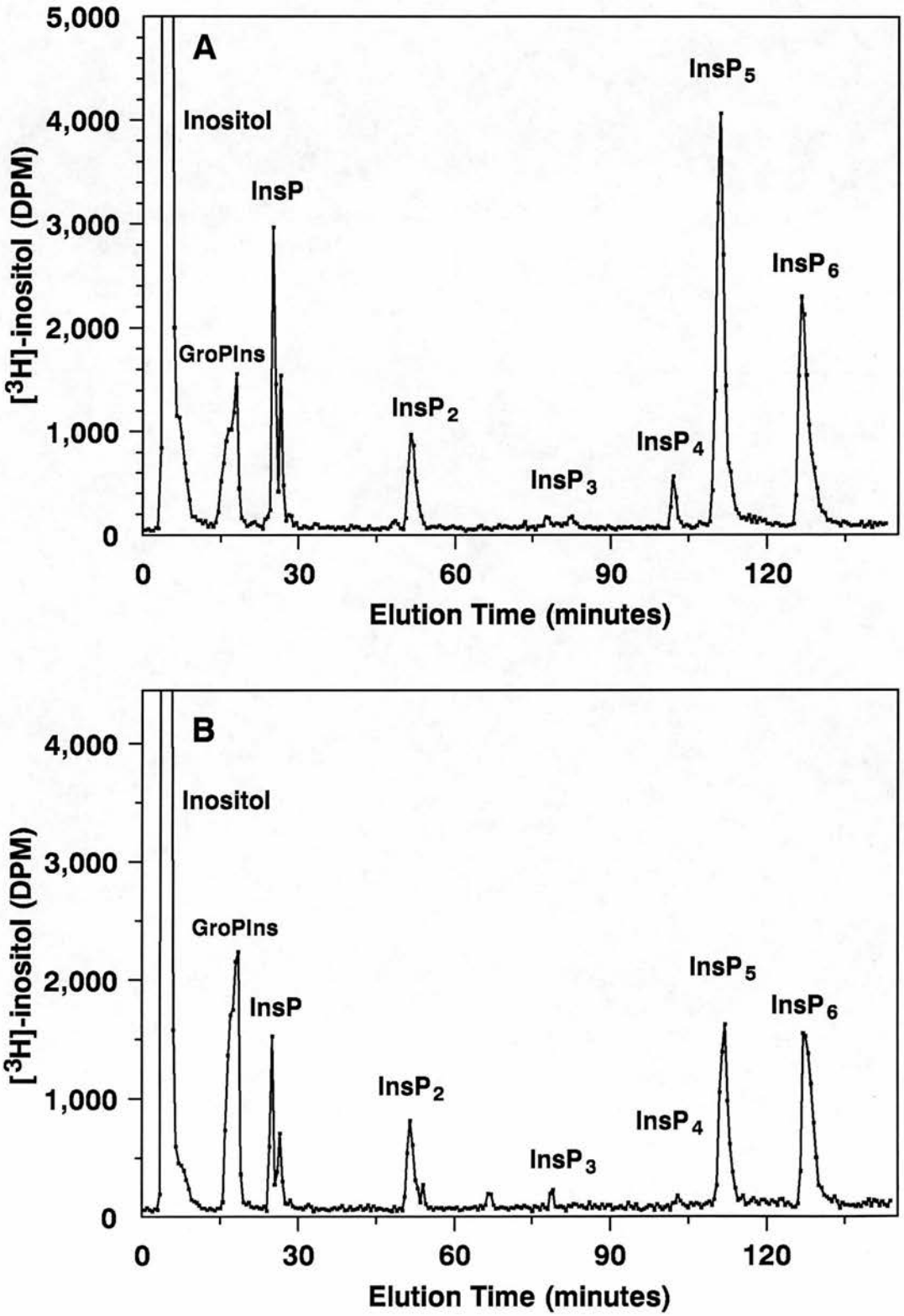
15.2. Changes in concentrations of inositol metabolites within normal myeloid blast cells during their differentiation towards monocytes.

In view of the documented changes that occur in intracellular concentrations of certain inositol polyphosphates during monocyte and neutrophil induced differentiation of HL60 cells (French et al, 1991), a comparative study was undertaken using normal myeloid blast cells. Undifferentiated myeloid blast cells purified following elutriation were cultured in serum-free medium containing 100 U/ml IL-3, 1 mg/l inositol and 2 μCi/ml [^{3}H]-inositol. After 5 days in culture, during which time equilibrium labelling with [^{3}H]-inositol had been achieved, cells were treated with 10 nM PMA. After a further 24 hours cells were harvested for analysis of total intracellular inositol metabolites. Control cells not treated with PMA were harvested at the same time for comparative study.

The efficacy of the PMA-induced monocyte differentiation of these cells was confirmed in each experiment by the microscopic examination of cultures. All PMA-treated cultures contained a considerable proportion of cells which had become pleomorphic, adherent and which were clumped as had been previously observed in PMA-treated cultures described in Chapter 13. Cells with these features, which are consistent with cells having undergone macrophage differentiation, were much less evident in control cultures. Furthermore, in three of the experiments cytospin preparations of cells were made from control and PMA-treated cultures and stained with May-Grunwald-Giemsa. Examination of these cytospins confirmed that promonocytic cells were present in much higher numbers after treatment with PMA for 24 hours than in control cultures. In analysis of these 3 experiments the mean values obtained for the percentage of cells showing evidence of promonocytic differentiation in cultures after 24 hours exposure to PMA was 48% whereas in control cultures the corresponding value was 21%. Granulocytic differentiation was also inhibited in PMA treated cultures. The mean of values obtained for the percentage of cells showing evidence of granulocytic differentiation in cultures after 24 hours exposure to PMA was 6% whereas the corresponding value for control cultures was 30%. Therefore, these data confirm the efficacy of PMA induction of monocyte differentiation of normal myeloid blast cells as described in Chapter 13.

In order to conserve cells for the study of intracellular inositol metabolites further characterisation studies of PMA induction experiments were not undertaken. Following treatment of blast cells with PMA there were changes in the concentrations of certain intracellular inositol metabolites when compared with control cultures. Figure 31 shows the HPLC traces obtained from a typical analysis of cells from both control and PMA-treated cultures. Table 24 shows the data obtained for total intracellular concentrations of inositol metabolites within cells from control and PMA-treated cultures. The major visible changes between the HPLC traces obtained for cells from control and PMA-treated cultures were the changes observed in the Ins P_4, Ins P_5 and putative GPI peaks. In the case of cells from PMA-treated cultures the Ins P_4 peak was barely visible and the level of the Ins P_5 peak was

Figure 31. HPLC Separation of the inositol metabolites within normal human myeloid blast cells in control (A) and PMA-treated (B) cultures.



reduced by approximately 50%. The peak provisionally labelled GPI (or GroPIns) was larger in the traces obtained for cells from PMA-treated cultures. The means of the values obtained after exposing cells for 24 hours to PMA for total intracellular concentrations of inositol metabolites are listed in Table 24.

Table 24. Changes in the intracellular concentrations of inositol metabolites during monocyte differentiation in normal human myeloid blast cells.

Inositol metabolite[1]	Concentration (μM) mean (±SEM) Control Cells	PMA treated cells[2]
Inositol	2610 (1200)	2030 (761)
Glycerophospho-inositol(GPI)[3]	22.4 (2.1)	73.2 (17)
Ins P	16.2 (2.1)	14.8 (1.4)
Ins P_2	9.4 (4.6)	12.9 (1.2)
Ins P_3[6]	ND	ND
Ins P_4[4]	4.2 (0.7)	1.1 (0.4)
Ins P_5[5]	37.2 (2.7)	20.7 (3.5)
Ins P_6	30.5 (5.1)	27.2 (3.5)

1. Inositol phosphates are labelled Ins P to Ins P_6 and values represent total concentrations of inositol phosphate isomers.
2. Cells treated with 10 nM PMA for 24 hours; number of experiments = 4.
3. P = 0.04, 2 sample paired T test.
4. P = 0.01, 2 sample paired T test.
5. P = 0.02, 2 sample paired T test.
6. ND = not determined.

There was no significant difference between control and PMA-treated cells in their total intracellular concentrations of unchanged myo-inositol. The intracellular concentration of inositol remained at high levels. The concentration of putative GPI was higher in cells from PMA-treated cultures than in control cells ($p = 0.04$, 2 sample paired T test). The concentrations of Ins P and Ins P_2 were not significantly different between cells from control and PMA-treated cultures. The Ins P_3 peak contained minimal numbers of disintegrations per minute in the case of cells from both control and PMA-treated cultures and therefore the concentration of Ins P_3 could not be accurately quantified. As shown in Figure 31, there were clear changes in the concentrations of Ins P_4 and Ins (1,3,4,5,6) P_5 when blast cells were treated with PMA. At 24 hours, the concentration of Ins P_4 in cells from PMA-treated cultures was 1.1 μM as compared with a value of 4.2 μM obtained for control cells ($p = 0.01$, 2 sample paired T test). The concentration of Ins (1,3,4,5,6) P_5 was reduced by 44% following PMA treatment of blast cells ($p = 0.02$, 2 sample paired T test). The concentrations of Ins P_6 within PMA-treated and control cells were not significantly different. Therefore, following treatment of undifferentiated myeloid blast cells with PMA for 24 hours there were significant reductions in the intracellular concentrations of Ins P_4 and Ins (1,3,4,5,6) P_5 and an increase in the inositol metabolite provisionally identified as glycerophospho-inositol. The significance of these findings and the comparison of these data with that generated from the study of HL60 cells are discussed later.

CHAPTER 16.
STUDIES ON A CASE OF PRIMARY MYELOFIBROSIS

An individual case of primary myelofibrosis was selected for further study for two reasons; firstly, to determine the potential of such patients as a source of haemopoietic precursor cells to develop model systems which can be used to characterise biochemical and molecular events associated with lineage commitment, proliferation and differentiation in the human haemopoietic system; and secondly to confirm clinical and morphological features in the patient suggesting an intrinsic defect in erythropoiesis and by further karyotypic and molecular study to determine the possible chromosomal location of genes which encode key regulators of erythroid lineage development. The clinical, haematological and morphological features of the case are described in more detail in the material and methods section (p61).

16.1 Immunophenotypic analysis of peripheral blood mononuclear cells.

The patient with primary myelofibrosis was studied with respect to cytogenetic analysis of peripheral blood cells and characterisation of peripheral blood mononuclear cells separated following ficoll-hypaque fractionation of whole peripheral blood. The initial studies included assessment of the MGG differential, the expression of cell surface antigens and the ability of cells to form colonies when plated in semi-solid media. When the initial studies were undertaken the patient's full blood count was as follows: Hb = 11.4 g/dl (post transfusion), WBC = 7.9 x 10^9/l and platelet count = 169 x 10^9/l. The differential white cell count was lymphocytes 1.2 x 10^9/l, monocytes 0.6 x 10^9/l, neutrophils 5.2 x 10^9/l, basophils <0.1 x 10^9/l, myelocytes 0.6 x 10^9/l, metamyelocytes 0.2 x 10^9/l and blast cells 0.2 x 10^9/l. Nucleated red cells were not seen on this occasion in the peripheral blood. Following ficoll-hypaque fractionation of 40 mls of peripheral blood, 15% of the leucocytes were recovered. The MGG differential of the mononuclear cell population was as follows: lymphocytes 48%, granulocytic cells (cells from promyelocyte to neutrophil stages of differentiation) 25%, monocytes 13%, blast cells 10%, normoblasts 0%

and unidentified cells 4%. The results obtained following immunophenotypic analysis of this population are shown in Table 25.

Table 25 Immunophenotypic analysis of peripheral blood mononuclear cells isolated from a patient with primary myelofibrosis.

	Antigens	% cells positive[1]
Myeloid	CD11b	25
	CD11c	19
	CD15	21
	CD14	15
Lymphoid	CD3 (T cell)	35
	CD19 (B cell)	11
	CD10 (B cell progenitor, mature granulocyte)	16
Other	CD7 (T cell, T cell/ myeloid precursor)	53
	HLA class II (progenitors, B cells, macrophages)	38

1. Percentages of positive cells were determined by use of the direct rosette technique.

The percentages of positive cells for the mature myeloid antigens CD11b, CD11c, CD15 and CD14 were in the range of 15 - 25%. These values were consistent with the percentages of granulocytic cells (25%) and monocytic cells (13%) observed following staining of the same cell population with MGG. The percentages of mature lymphocytes (43%) as assessed by the combination of CD3 (T cell) positive (32%) and CD19 (B cell) positive (11%) cells also showed good agreement with the percentage of lymphocytes observed following staining with MGG

(48%). The values for CD3 positive cells (32%) and CD5 positive cells (35%) were in close agreement. Both antigens are markers of mature T cells but CD5 does recognise, in addition a subset of B cells. The percentage of CD10 positive cells was 16%. The discrimination between CD10 positive B cell progenitors and CD10 positive mature granulocytes was not further elucidated. Other antigens studied included the CD7 and class II HLA antigens. These antigens are known to be expressed on both mature and precursor haemopoietic cell populations. For example HLA class II antigen expression is a feature of B cells and macrophages and also of progenitor cells. CD7 antigen expression is a feature of mature T cells and also T cell and myeloid precursors. Both these antigens were expressed to a higher degree than the percentages of the corresponding mature cell populations present, indicating that a significant population of precursor cells was present in the fractionated cell population. The finding of 10% blast cells in the MGG differential supported this observation.

These data on the characterisation of the fractionated mononuclear cell population by MGG differential and immunophenotypic analysis indicated that the cell population consisted mainly of mature myeloid and lymphoid cells and that the haemopoietic precursor population constituted only a small minority of cells. Studies of the cytogenetic abnormalities and colony forming ability of peripheral blood mononuclear cells isolated from this patient were undertaken and are described below.

16.2 Colony forming ability of peripheral blood mononuclear cells.

Fresh peripheral blood mononuclear cells isolated from the above patient were assayed for their ability to form erythroid and granulocyte macrophage colonies in semi-solid medium. The results are shown in Table 26 and are expressed as CFU/ml of peripheral blood. Similar results were demonstrated on 3 different occasions which revealed greatly increased numbers of circulating CFU-GM with the complete absence of erythroid colonies. The nature of GM colonies was confirmed following staining of cytocentrifuge preparations of sample single colony contents. Positive control erythroid colonies were grown

in appropriate numbers from fractionated human fetal liver cells (Toksoz and Brown, 1984) and from normal bone marrow cells, indicating that the failure to detect erythroid progenitors was not due to technical reasons.

Table 26 Number of colony forming cells grown from PMF peripheral blood mononuclear cells and from normal cells.

	PMF PBMNC		FETAL LIVER		BONE MARROW	
	CFU/ml of blood		CFU/10^5 cells			
Time Point	CFU-GM	BFU-E/CFU-E	CFU-GM	BFU-E	CFU-GM	BFU-E
1	2,644	0	103	153	33	22
2	7,912	0	122	155	70	28
3	7,605	0	300	90	27	ND[1]

1. ND = not determined.

16.3 Cytogenetic studies

Cytogenetic analysis of peripheral blood cells from the patient with myelofibrosis was undertaken to detect abnormalities present in haemopoietic cells. Analysis of 20 metaphases from unstimulated cultures of peripheral blood cells revealed 3 cell lines: 1 metaphase showed a normal male karyotype: 13 cells showed 46 chromosomes with the loss of one chromosome 2 and one 11, the presence of an abnormal chromosome 2 derived from a 2;11 translocation and an additional unidentified marker chromosome (46, XY, -2, -11, +der(2) t (2;11) (q24/31;q13), + mar); and 6 cells showed the above karyotype along with the loss of one chromosome 17 and a deletion of part of the long arm of one chromosome 7 (45,XY,-2,-11, +der(2)t (2;11)(q24/31;q13), +mar,-17,del(7q)). This complex karyotypic abnormality has not been reported previously in primary myelofibrosis.

Pooled GM colonies grown from peripheral blood mononuclear cells were also subjected to karyotypic analysis. Analysis of the progeny of pooled colony forming cells showed the third karyotype described above in 8/10 metaphases. The other two metaphases described the same karyotype with the addition of another unidentified marker chromosome, implying some further clonal evolution (Plate 8).

The primary fibroblast cultures harvested from a skin biopsy from the above patient were also subjected to karyotypic analysis. Analysis of all these metaphases revealed a normal male karyotype. This evidence supports the fact that the patient exhibited a normal constitutive karyotype and had acquired an abnormal karyotype in haemopoietic lineages in association with the development of primary myelofibrosis. Epstein-Barr virus transformed B cell lines were also generated from this patient (in collaboration with Dr C Gregory, Department of Immunology) and then subjected to karyotypic analysis to determine whether any of the cell lines isolated carried the same karyotypic abnormality as the patient's other haemopoietic cells. Twenty lymphoblastoid cell lines were derived and cloned from this patient. Cells from all the lines, designated EBoy 1-20, exhibited a normal male karyotype. This last observation suggested that either 1) the clonal disorder giving rise to myelofibrosis did not involve B lineage precursors or 2) the more likely possibility that the lymphoblastoid cell lines had been generated from long lived B cells that had themselves been generated from normal B cell progenitors.

The patient's peripheral blood cells were further studied at the terminal stage of his disease. A more complex karyotype had evolved which was 45, XY, t(1p,18p),-2,-11,+der(2)t(2;11),-4,del(7q),-16,+der(16)t(16;?), -17,+mar1,+mar2.

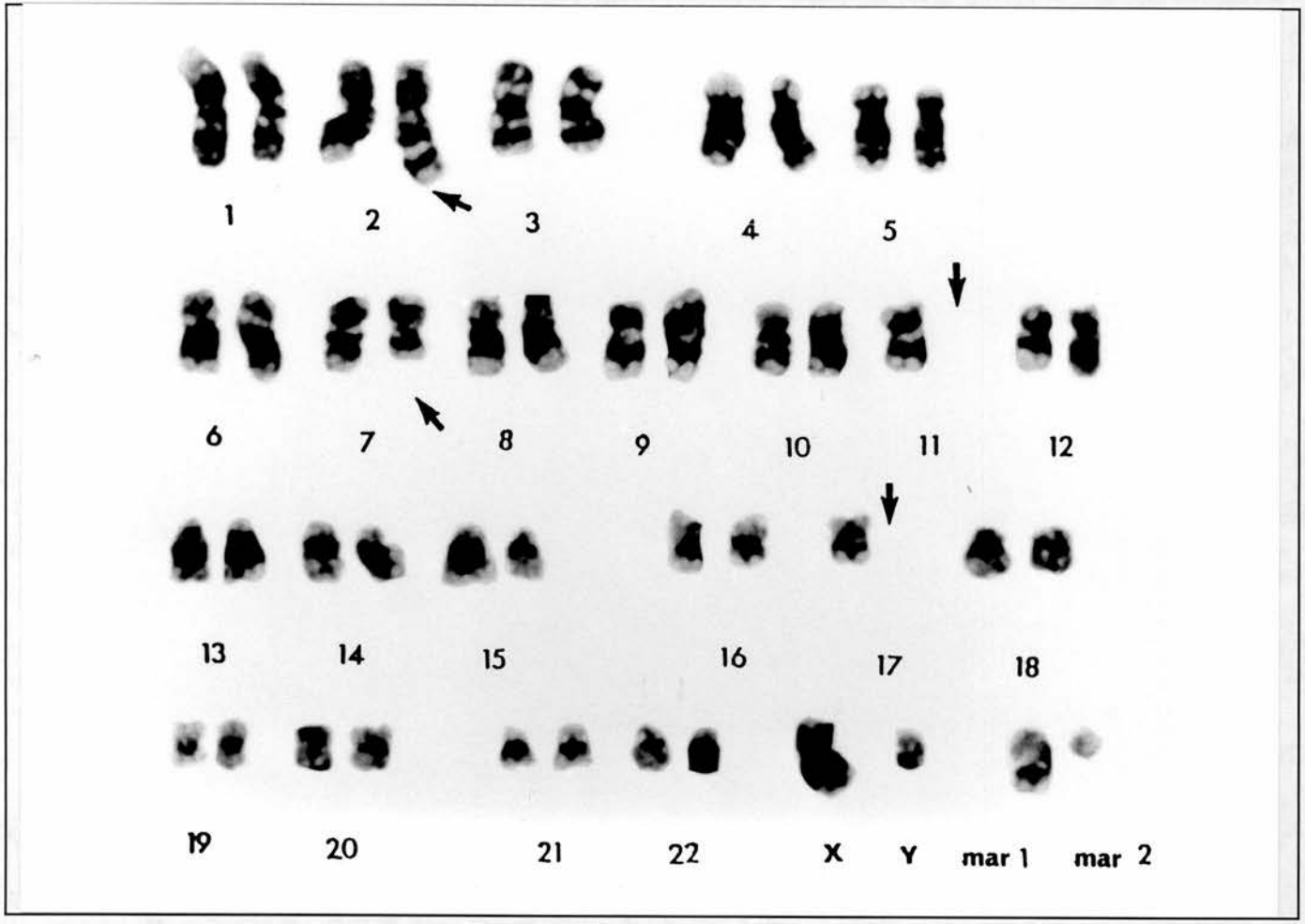


Plate 8. G banded karyotype from pooled GM colony culture from the patient with primary myelofibrosis: 46, XY, -2, -11, + der (2) t (2;11) (q24/31;q13), + mar 1, -17, del (7q), + mar 2.

16.4 Initiation of haemopoiesis by PMF peripheral blood mononuclear cells on irradiated stroma grown from long term bone marrow cultures.

The finding of absent circulating erythroid progenitor cells in PMF is an uncommon event, which in the patient described above may have been due to an intrinsic defect in the capacity of haemopoietic stem cells to undergo commitment to the erythroid lineage or the ability of committed erythroid progenitors to undergo erythropoiesis. Previous studies have shown that in PMF patients, CFU-GM can be maintained in liquid suspension culture of peripheral blood mononuclear cells in the absence of a substantial stromal layer over a 10 week period, indicating that primitive stem cells may circulate in PMF patients (Douay et al, 1987). In the following experiment fresh peripheral blood mononuclear cells harvested from the patient with PMF were co-cultured with irradiated, allogeneic normal bone marrow stroma (Plate 9) to maintain circulating primitive stem cells and to assess whether they were able to give rise to erythroid progenitors in a normal and appropriate microenvironment. Cultures were established which contained 1) irradiated normal bone marrow stroma alone; 2) peripheral blood mononuclear cells from the patient with PMF alone and 3) Co-cultures which contained peripheral blood mononuclear cells from the patient with primary myelofibrosis and irradiated allogeneic normal bone marrow stroma. After 5 weeks in culture the cells were harvested and assayed for CFU-GM and BFU-E numbers. These results are shown in Table 27.

Table 27. Mean number (± SEM) of CFU isolated after culture of PMF PBMNC on irradiated bone marrow stroma.

Culture condition	CFU-GM		BFU-E/CFU-E
	per 10^5 cells	per total culture	
stroma alone	0	0	0
PMF cells alone	14(1.1),11(1.7)	156(12), 101(16)	0
PMF cells + stroma	27(2.1), 20(0.7)	405(31), 348(11)	0

9a. 9b. 9c.

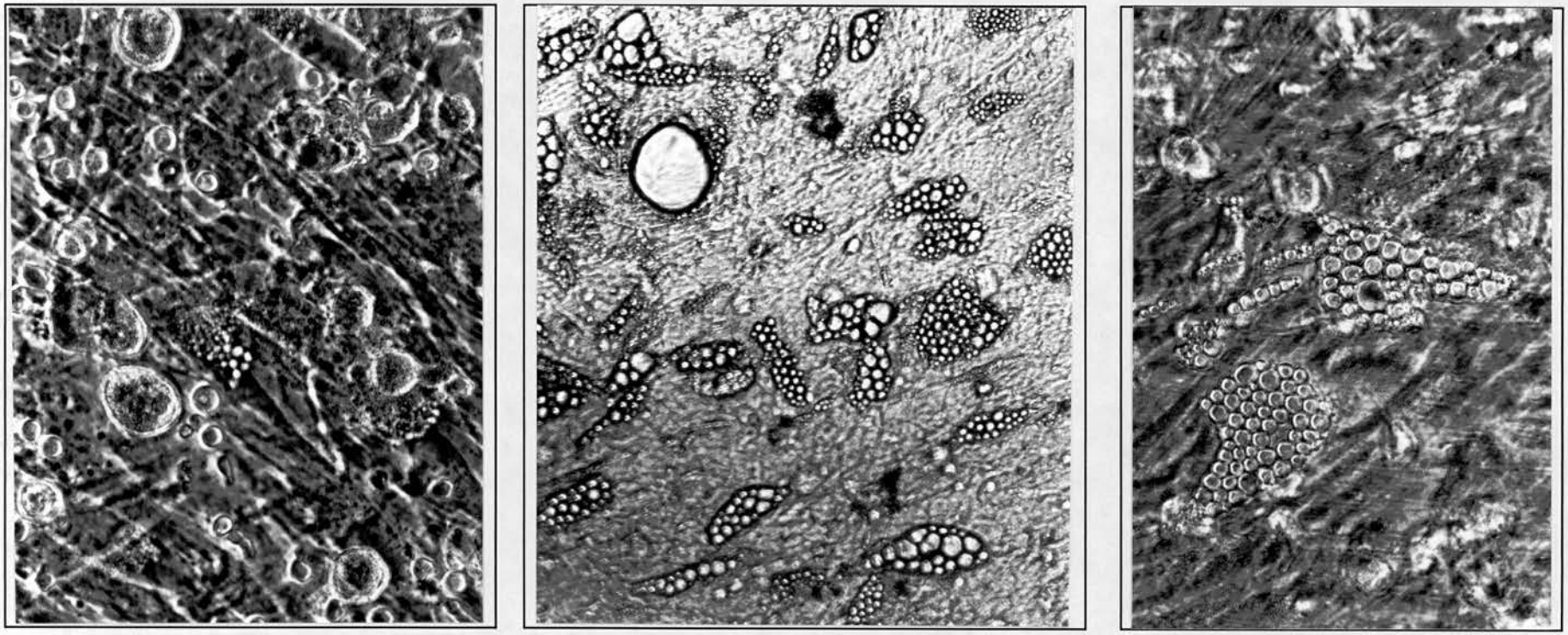


Plate 9. Photomicrographs from representative areas of confluent stroma grown from long term cultures of normal adult human bone marrow. Plate 9a shows an area containing predominantly fibrobasts and monocytes; Plate 9b shows an area rich in giant adipocytes; and Plate 9c shows an area containing foci of haemopoietic cells known as cobblestone areas (x 125).

No GM or erythroid colonies were grown from the culture containing irradiated allogeneic normal bone marrow stroma alone. In cultures containing PMF peripheral blood mononuclear cells only GM colonies were isolated. However, co-cultures containing both irradiated stroma and PMF peripheral blood mononuclear cells yielded much greater numbers of GM colonies than cultures containing PMF PBMNC alone whether numbers were expressed in terms of total CFU per culture or per 10^5 inoculating cells. When the patient's cells were co-cultured with normal allogeneic bone marrow stroma, the average values (+SEM) for the numbers of GM-CFC generated per culture in two experiments were 405 (31) and 348 (11) respectively. However, when the PMF PBMNC were cultured alone the corresponding values for the numbers of GM-CFC per culture were 156 (12) and 101 (16) respectively. No erythroid colonies were grown from these cultures. MGG stained cytocentrifuge preparations of cells taken from cultures at the time of clonogenic assay did not show any cells of the erythroid lineage. The finding of increased numbers of CFU-GM following incubation of PBMNC with marrow stroma as compared with control PBMNC cultured in the absence of stroma supported the existence of circulating stem cells in PMF and the inability to generate erythroid progenitor cells in this culture system further supported an intrinsic defect of erythropoiesis in this case.

16.5 Determination of the involvement of pSEA locus in the t(2;11) translocation noted in this patient.

The proto-oncogene pSEA, which is the human homologue of the viral oncogene SEA of the S13 avian erythroblastosis virus, maps to the region of 11q13 which was involved in the chromosomal translocation noted in PBMNC isolated from this patient with PMF. As this case was associated with defective erythropoiesis it was of interest to study the potential involvement of the proto-oncogene in the t(2:11) translocation observed in this patient. This was performed by comparing Southern blots of DNA which had been prepared from sources of both normal (skin fibroblast) and abnormal (PBMNC) DNA and which had been hybridised with a ^{32}P labelled cDNA probe to pSEA. Three different restriction endonucleases were employed to digest the DNA. The

autoradiographs of the hybridised Southern blots are shown in Plate 10. The pattern of bands visualised was identical using DNA derived from either peripheral blood mononuclear cells or skin fibroblasts. This suggests that the pSEA locus has not been disrupted by the chromosomal translocation.

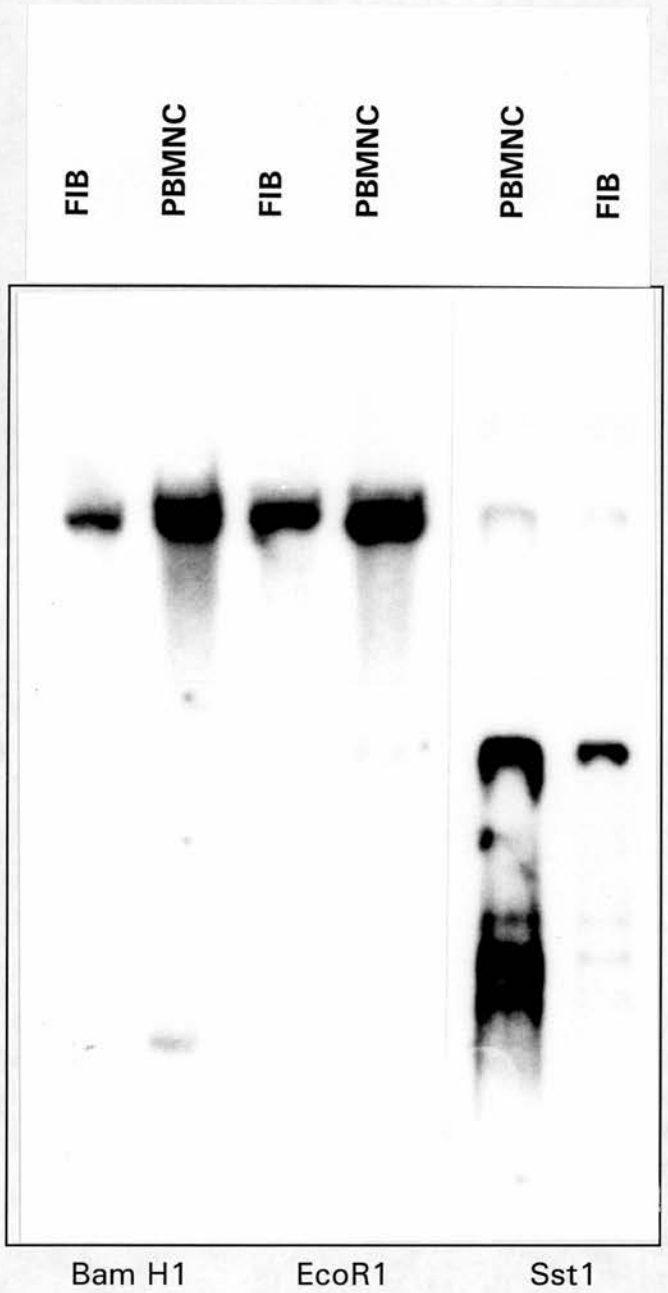


Plate 10. Southern blot analysis of DNA obtained from peripheral blood mononuclear cells (PBMNC) and skin fibrolasts (FIB) isolated from the patient with primary myelofibrosis. The DNA was digested with BamH1, EcoR1 and Sst1 restriction endonucleases and the filters were hybridised with a 4.5kb cDNA probe to pSEA.

SECTION 4. DISCUSSION

CHAPTER 17. STUDIES USING BLAST CELLS PURIFIED FROM HUMAN FETAL LIVER

The principal objective of this thesis was to purify normal human myeloid cells at a very early stage of differentiation in sufficient numbers for the subsequent study of intracellular biochemical events related to myeloid differentiation. Such a model system would permit comparative studies with abnormal cells or cell lines to be undertaken which might delineate events pertinent to malignant transformation. Human fetal liver, which is known to be a rich source of haematopoietic progenitor cells (Champlin and Gale 1980; Toksoz and Brown 1984; Emerson et al 1985), was used as the source of haemopoietic cells and a population of undifferentiated myeloid blast cells, with a similar differentiation status to HL60 cells, was purified. Treatment of the blast cells with PMA was shown to promote rapid monocyte differentiation and to inhibit spontaneous granulocyte differentiation which was present in control cultures of these cells. Studies of intracellular inositol metabolites within undifferentiated normal myeloid blast cells and changes during PMA-induced monocyte differentiation of these cells were then performed and the results were compared with those obtained from previous studies performed using HL60 cells. The observations suggest a possible, but as yet unidentified, role for inositol polyphosphates in myeloid differentiation. The ability to purify homogeneous populations of undifferentiated normal haemopoietic cells for biochemical and molecular studies of differentiation is a significant achievement.

It was very important in this study to aim to purify undifferentiated haemopoietic cells from fetal liver using negative selection techniques. Negative selection methods for enriching cell populations have the advantage that the required cell population is not directly stimulated by binding of antibodies to their surface and are therefore unperturbed. This approach was preferred over positive selection methods since the cells were required for in vitro biochemical studies. The positive selection of cells by antibody labelling may interfere with cells' physiological properties by effecting intracellular biochemical changes

occurring as a result of antibody binding or alternatively, antibody bound to the cell surface has the potential to interfere stoichally with the binding of normal external factors. Furthermore, the positive selection of cells by rosetting with $CrCl_3$-coupled erythrocytes and the negative selection of cells using antibody combined with complement mediated lysis have not been recommended because of non-specific damage to the desired population of haemopoietic precursor cells (Emerson et al, 1985).

In this study cells were initially purified following density separation and unwanted cells were removed after coating these cells with a mixture of two monoclonal antibodies which identify glycophorin C and a monocyte associated antigen respectively. The subsequent separation of cells by counterflow cell elutriation (CCE) allowed further purification of primitive cells without direct interference with the desired cell population. Thus, the blast cells obtained were not subjected to either antibody coating or possible damage by $CrCl_3$-coupled erythrocytes. Furthermore, since the fetal liver cells were first purified as light density cells using ficoll-hypaque and then by subsequent negative selection using ficoll-hypaque, the initial fractionation procedure depended only on the criterion of antigen expression. As an alternative, negative selection by panning out the unwanted cell populations has been recommended for the isolation of haemopoietic progenitor cells from human fetal liver (Emerson et al, 1985). However in these circumstances, when antibody coated cells adhere to plastic surfaces coated with anti-immunoglobulin, both antigen expression and non-specific adherence are factors involved and the process may selectively remove some adherent myeloid blast cells of interest. A minimal combination of two antibodies was used to coat unwanted cell populations to avoid the possibility that the combined effects of a large cocktail of monoclonal antibodies, each binding to haemopoietic cell surface antigens expressed at uncertain and/or low levels on undifferentiated myeloid blast cells, may generate indirect rosettes and eliminate some cells of interest.

Previous studies describing the growth and differentiation in liquid suspension culture of mononuclear cells isolated from human fetal liver (Toksoz and Brown, 1984) had shown that these cultures generated

large numbers of mature neutrophils and macrophages and sustained GM-CFC for 4 weeks indicating that the initial cell population contained cells at early stages of myeloid differentiation. In this study the cell population obtained from fetal liver following mechanical disruption of tissue, density gradient fractionation and rosette depletion of unwanted cells (Chapter 11.1, p95) was composed mainly of undifferentiated myeloid blast cells and a few containing lymphocytes and more mature myeloid cells (table 12, p103). The purified blast cells lacked features of differentiation such that they could not be identified as either precursors of neutrophils or monocytes (Plate 1b, p 104). When cultured in medium containing fetal calf serum these cells differentiated towards either neutrophils or macrophages. However, as reported previously for the light density fetal liver cells (Toksoz and Brown, 1984), the terminal differentiation of the population as a whole occurred over a protracted time course. Both the slow changes in the differentiation status of the population and the fact that both granulocytes and monocytes are generated, presented difficulties in using these cells to analyse biochemical events pertinent to either neutrophil or macrophage differentiation.

The phorbol ester, PMA is known to induce the rapid terminal differentiation of various cell types, including keratinocytes (Parkinson and Emerson, 1982) and rapid differentiation of the human promyeloid cell line, HL60 towards monocytes (Rovera et al, 1979). When PMA was added to cultures of undifferentiated blast cells purified from human fetal liver (fig. 22, p136), 62% of the cells differentiated towards macrophages within 42 hours as revealed by increased numbers of cells with an appropriate morphological appearance and by cells which expressed the enzyme α-naphthyl acetate esterase. Thus the addition of PMA provided a reduced time course for the differentiation of a population of the blast cells towards macrophages. An unexpected finding was that PMA inhibited granulopoiesis within the cultures, possibly by affecting the 31-51% of blast cells which underwent neutrophil differentiation in the control cultures. This inhibition of granulopoiesis in the PMA-treated cultures was revealed by the lack of increased numbers of cells at or beyond the promyelocyte stage of differentiation (AGF4.48 positive) and by the lack of cells expressing

lactoferrin (fig. 23, p141.). The studies of other cytochemical markers (fig 24, p144) and the expression of other leucocyte surface antigens (table 22, p146) confirmed the finding that PMA promoted the rapid monocyte differentiation of the undifferentiated myeloid blast cells purified from human fetal liver whilst concomitantly inhibiting granulocyte differentiation. The practical benefit from the rapid promotion of monopoiesis and simultaneous inhibition of granulopoiesis is that a population of normal cells is provided which is undergoing macrophage differentiation only. This is advantageous to studies of biochemical events during monopoiesis since, as suggested above, a mixture of blast cells undergoing macrophage differentiation and neutrophil differentiation would display events pertinent to both pathways of maturation but which could not be studied in isolation. An alternative solution to this problem would be to separate the two blast cell populations. However the means to achieve this are not presently available.

The capacity of PMA to promote monopoiesis and inhibit granulopoiesis has important implications in relation to intracellular processes which regulate these two pathways of differentiation. The purified blast cell population is most likely to be a mixture of cells which are already committed to either neutrophil or macrophage differentiation, since a maximum number of 65% of the cells were induced to mature towards monocytes by PMA and in control cultures 34% of the cells were able to spontaneously differentiate towards neutrophils. The 34% of cells able to differentiate towards neutrophils correlates well with the percentage of blast cells which did not differentiate in PMA-treated cultures (32%). Thus, PMA would not appear to interfere with granulopoiesis by diverting cells towards the monocyte pathway of maturation. This conclusion leads to the view that the opposing effects of PMA on granulopoiesis and monopoiesis are related to a reciprocal interaction between intracellular processes which regulate the capacities for the two pathways of maturation. In other words, intracellular events effected by PMA which facilitate monopoiesis concomitantly lead to a reduced capacity for granulopoiesis. For example, PMA is known to activate protein kinase C (PKC) which may phosphorylate proteins and confer functional activity which is essential for monocyte differentiation.

As irreversible commitment of progenitor cells to a particular pathway of differentiation is involved, these proteins would presumably include DNA-binding proteins, the PMA-responsive elements identified as modulators of gene activity in several cell types (Lee et al, 1987). The reciprocal enhancement and silencing of genes required for monocyte and neutrophil differentiation respectively in this system, could thus be achieved through activation of the PKC pathway and the ultimate modulation of specific DNA-binding proteins.

The above general consideration as to the effect of PMA also accords with previous proposals that expressions of the potentials for both neutrophil and monocyte differentiation are closely linked. Brown et al, 1985 have interpreted data from various studies to suggest that the above two differentiation potentials are expressed in a progressive and linear manner, in that as cells lose the capacity for neutrophil differentiation they then acquire the potential for monocyte differentiation. If this hypothesis is correct, then only one or two choices are available to developing haemopoietic progenitor cells at any given time and lineage potentials can be viewed as sequential partially overlapping differentiation options (p 11; Brown et al, 1989). The notion that PMA promotes monopoiesis and inhibits granulopoiesis by virtue of a reciprocal interaction between intracellular events, which regulate commitment to these two pathways of differentiation, provides a mechanism whereby regulation of differentiation potentials can be achieved. Further support for such a model, that lineage potentials are sequentially expressed, can be proposed as follows. At the molecular level, the modulation of gene expression required for all diversification may be effected via a relatively small group of transcriptional regulators, governed by the major intracellular signalling pathways, including protein kinase C. Specificity is introduced at the level of modulation of the activity of these gene regulators and the accessibility of these gene loci. The minimal activation of one signalling pathway, leading to the enhancement and silencing of particular genes, would necessitate the availability of only two differentiation options as regards the expression of lineage potentials. In contrast, a multipotent model of haemopoiesis in which cells can differentiate directly along any one of at least five maturation pathways (Heyworth et al, 1988), is likely to require the

involvement of several intracellular signalling pathways, which modulate a panel of DNA-binding proteins, so as to select one differentiation option and close down the others.

In parallel to the studies of the effects of PMA on the differentiation of purified blast cells, the purified blast cells were further characterised with respect to their cytochemistry, the expression of cell surface antigens and their ability to form colonies when plated in semi-solid medium. (Ch. 12.1, p120). The pattern of cytochemical staining observed did confirm that this was a population of cells showing limited evidence of myeloid differentiation. However previous staining with MGG did show that the cells exhibited variable morphological features (p104) and indicated that they were not an entirely homogeneous population. Analysis of their colony forming ability indicated that only a small minority of cells (0.5%) were able to give rise to colonies under the culture conditions used in the assay. Immunophenotypic analysis of the purified undifferentiated blast cells did confirm that the cells were haemopoietic in nature as 99% of the cells reacted with the antibody to common leucocyte antigen. The percentage of cells expressing glycophorin C, an erythroid antigen, was low (1%) illustrating the efficiency of the erythroblast depletion procedure. The percentages of cells positive for the mature myeloid antigens CD11c, CD15 and CD14 were all low (<8%) and these values were consistent with the findings observed following cytochemical and MGG staining. The number of mature T cells present, as identified by CD3 staining, was also low (1%) but a significant percentage of cells were positive for the CD10 (18%) and CD19 (14%) antigens indicating that B cell progenitors were present within the purified population. Very much larger percentages of cells were positive for HLA Class II (59%), CD7 (46%) and CD34 (32%) antigens indicating that the population consisted largely of undifferentiated haemopoietic cells.

However, to enhance the yield of undifferentiated blast cells obtained from each fetal liver the purification procedure was modified to incorporate initial enzyme digestion of fetal liver tissue, susbsequent ficoll-hypaque separation of light density cells, which was performed twice, and finally rosette depletion of fetal liver cells using the Ret40F

and 61D3 monoclonal antibodies (Ch 11.3, p111). The enzyme digest procedure did result in significantly increased cell yields from each fetal liver but the new procedure also highlighted qualitative differences between the mononuclear cell populations obtained when using the two purification methods. In addition to an increase in cell yields, the percentages of normoblasts and macrophages were considerably higher in interface populations obtained following enzyme digestion of fetal tissue. For example, following enzyme digestion and the second ficoll-hypaque fractionation the percentage of normoblasts in the interface population was 55% whereas this value was 27% in the interface population obtained following mechanical disruption of fetal tissue; following enzyme digestion and the second ficoll-hypaque fractionation the percentage of monocytes and macrophages in the interface population was 11.3%, whereas this figure was only 2.2% in the interface population obtained following mechanical disruption of fetal tissue. The modified method therefore, enhanced total cell yields but also enriched for the proportion of fragile normoblasts and adherent macrophages possibly because they were not removed in a cotton wool filtration step.

This qualitative difference in the purified populations obtained using the two different methods was also apparent following the rosette depletion step. For the same reasons as outlined above, the final purified population obtained after enzyme digestion and rosette depletion using Ret40F and 61D3 contained a much higher proportion of promonocytes (13.9%) than the final population obtained following mechanical disruption of tissue (4.6%). The percentage of mature macrophages was low in both purified populations indicating the efficiency of the rosette depletion procedure in removing mature macrophages but this figure was again slightly higher in the enzyme digest population (0.5%) than in the mechanical disruption population (0.1%). Mainly as a result of the increased proportion of promonocytes, the enzyme digested final purified population contained fewer undifferentiated blast cells (59.2%) than the population obtained following mechanical disruption of tissue (76.5%). However despite the lower percentage value for undifferentiated blast cells, the enzyme digestion method produced a far greater yield of cells from each fetal liver (7.3×10^6 cells v 3.6×10^6

cells) than that obtained following mechanical disruption of tissue. Therefore the modified procedure gave approximately twice the number of blast cells than obtained previously using mechanical disruption.

In an attempt to obtain a more homogeneous blast cell population from the enzyme digested liver material the 61D3 and Ret40F depleted mononuclear cells were further purified by counterflow cell elutriation (CCE). This procedure separates cells according to their size and buoyant density and has been successfully used to separate murine CFU-S from PHSC (Jones et al, 1990; Material and Methods p72). Following this procedure it was discovered that the collection and pooling of two CCE fractions obtained at flow rates of 13.3 and 17.8 ml/minute with a rotor speed of 1950 rpm gave the optimal combination of cell yield and separation of undifferentiated blast cells from other cell types including promonocytes and lymphoid progenitor cells. These fractions also contained the highest percentages and the greatest absolute numbers of cells positive for the CD34 antigen. These cells were also more homogeneous in nature with respect to their MGG staining characteristics (Plates 3 c-d, p117) than the blast cells observed prior to the elutriation step (Plate 2d, p110). Approximately 20% of the cells placed in the elutriation chamber were recovered in these two fractions. In five experiments a mean of 1.75×10^6 cells was recovered from each fetal liver representing a yield of 0.07% of the cells obtained following enzyme digestion of liver tissue.

The studies previously outlined indicated that the population of cells purified from human fetal liver consisted mainly of undifferentiated myeloid blast cells which were capable of maturing to become either granulocytes or monocytes. In this manner the cell population provides a new normal cell model system which is parallel to the human cell line HL60. HL60 is an undifferentiated myeloid cell line which is also capable of either granulocyte or monocyte differentiation. Previous studies had delineated changes in intracellular metabolites of inositol in association with PMA-induced monocyte differentiation of HL60 cells (French et al, 1991) and it was of interest to determine whether the same changes occurred during PMA-induced monocyte differentiation of undifferentiated normal myeloid blast cells. To perform these

experiments in comparable conditions to the HL60 cell experiments it was necessary to maintain the myeloid blast cells in an undifferentiated state in serum-free medium containing low amounts of inositol for a long enough period to permit equilibrium labelling of the inositol metabolites within the cells. Once the equilibrium labelling of inositol metabolites had been achieved, the changes in metabolism which occur when the blast cells differentiate in response to PMA could then be investigated. When placed in serum-free medium, as used in the earlier HL60 cell studies, the blast cells died rapidly. However, when IL-3 was incorporated into the culture medium (100 U/ml), the cells survived, increased in number and maintained an undifferentiated phenotype. The survival of these undifferentiated blast cells in high concentrations of IL-3 is consistent with the known factor dependence of early myeloid precursor cells and is analogous to the maintenance of the undifferentiated status of murine IL-3 dependent cell lines (Heyworth et al, 1990).

The labelling of blast cells in medium with a known specific activity of [^{3}H]-inositol and measurement of the mean cell volume permitted the determination of the concentrations of inositol metabolites within the cells. The levels of several inositol phosphates within undifferentiated normal myeloid blast cells were similar to those observed within the promyeloid cell line HL60 (French et al, 1991). The concentrations of Ins P, Ins P_2, Ins P_4 and Ins P_5 observed in undifferentiated normal myeloid blast cells and HL60 cells, respectively, were as follows: Ins P, 16μM versus 24μM; Ins P_2, 9.4μM versus 14μM; Ins P_4 4.2μM versus 4.4μM and Ins P_5 37μM versus 27μM. Other compounds displayed marked differences in concentration between the two cell populations. HL60 cells contained more Ins P_6 than the fetal liver blast cells (63μM versus 31μM) and less glycerophospho-inositol (GPI) (11μM versus 22μM). A particularly striking difference between the two cell populations was the concentration of free intracellular inositol. Within the undifferentiated normal myeloid blast cells the concentration of inositol was 2610μM which is much higher than the 39μM observed within HL60 cells. This difference may in part be explained by the fact that the normal blast cells were obtained from fetal tissue which has a high extracellular concentration of inositol in the surrounding fluids

(125μM, Lewin et al, 1978; Quirk and Bleasdale, 1983). In contrast, the HL60 cells had been maintained long term in medium containing much lower amounts of inositol (5μM). However the higher level for intracellular inositol within the cells as opposed to the external medium suggests that inositol is actively transported within both cell types. Differences in inositol pools may indeed reflect a different rate of inositol transport in each cell type. Also, the similar values for the higher inositol phosphates found in cells with different sized pools of free intracellular inositol suggests that cells actively control their levels of highly phosphorylated derivatives even when the level of intracellular inositol is restricted. This further supports the hypothesis that these compounds have an important role within cells.

The changes previously described in inositol phosphates during PMA-induced monocyte differentiation of HL60 cells included rapid and sequential declines in Ins P_4 and Ins P_5 and a slower fall in Ins P_6 (French et al, 1991). In HL60 cells treated for 24 hours with PMA there was a 75% decrease in the level of Ins P_4 and an 80% decrease in the level of Ins P_5. Similar changes in these two compounds were observed at 24 hours when the fetal blast cells had differentiated towards monocytes in response to PMA: Ins P_4 had declined by 75% and Ins P_5 by 50%. The slow decline in the level of Ins P_6 within HL60 cells was noted at 48 hours and was not significant at 24 hours. The fetal blast cells had been harvested 24 hours after the addition of PMA and therefore may not have reached the point when a change in the level of Ins P_6 might have been observed.

The significance of the decreases in the levels of both Ins P_4 and Ins P_5 which occurred in association with monocyte differentiation of both normal myeloid blast cells and HL60 cells remains to be determined. It is possible that these changes may be a key feature of cells undergoing monocyte maturation or alternatively they may represent epiphenomena related to the effects of treatment of cells with PMA. It would therefore be of particular interest to determine whether similar changes occur in normal myeloid blast cells as they undergo differentiation in response to physiological stimuli. This might be achieved by permitting cells to spontaneously differentiate in serum containing culture medium or by

the addition of M-CSF to the culture medium. However, as the blast cells are a mixed population of cells committed to neutrophil and monocyte differentiation, it is probable that such cultures would contain a mixture of mature granulocytes and mature monocytes. Therefore to perform the analysis of intracellular inositol phosphates one would have to separate the two populations of cells. It is not possible, at present, to separate the blast population into those committed to either granulocyte or monocyte differentiation but it may prove possible to separate more mature populations. For example, cells undergoing granulocyte differentiation begin to express the CD15 antigen on their cell surface at the promyeloid stage of maturation and cells expressing CD15 could be rosette depleted using sheep red cells or magnetic beads bound to an antibody directed against the CD15 antigen. This should leave behind a population of cells which are negative for the CD15 antigen and which should therefore be enriched with cells which have undergone monocyte maturation. This population of normal cells which have undergone physiological monocyte differentiation could therefore be used for analysis of intracellular inositol phosphates.

A complementary approach would be to study the effects of PMA on the intracellular inositol metabolites within variant HL60 cells which arrest their growth in response to PMA but which do not undergo monocyte differentiation (Bunce, 1987). If the same changes in Ins P_4 and Ins P_5 occur in these circumstances they must be related either to growth arrest or epiphenomena related to treatment of cells with PMA.

The reason for the changes in these inositol metabolites observed during PMA-induced monocyte differentiation remains unresolved. If these events are related to PMA, it is possible that the fall in Ins P_4 seen in both cell types is related to protein kinase C activation of the 5-phosphatase which degrades Ins (1,3,4,5) P_4, the likely major component of the Ins P_4 peak (Bansal and Majerus, 1990). However this 5-phosphatase also degrades Ins (1,4,5) P_3 and in the HL60 studies no depletion of Ins (1,4,5) P_3 was observed in response to PMA which therefore argues against this possible explanation for the depletion of Ins P_4.

The remaining major change which occurred as the normal myeloid blast cells underwent differentiation towards monocytes was a large increase in the putative glycerophospho-inositol (GPI) peak (from 22μM to 73μM). This was not observed as HL60 cells differentiated towards monocytes and the reason for this discrepancy between the two promyeloid cell populations during the differentiation towards monocytes is not clear. Glycerophospho-inositol is formed following the serial deacylation of phosphatdyl-inositol by the enzymes phospholipase A_2 (PLA_2) and a subsequent lysophospholipase. These steps result in the removal of the two fatty acid side chains of the glycerol backbone of phosphatidyl-inositol. An important difference between the cell populations might therefore lie in the control of membrane bound phospholipase A_2.

As these data have highlighted both similarities and differences between normal undifferentiated myeloid blast cells and HL60 cells it would also be of interest to study similar phenomena in cells from patients with acute myeloblastic leukaemia (AML). Such comparative studies by showing similarities in HL60 and AML cells but not in normal myeloid blast cells might indicate phenomena which directly relate to the cells' transformed status.

In this study recombinant human IL-3 was used to maintain the purified blast cells in serum-free medium. However, more recently other multipotent factors have become available and it is likely that the use of such factors will increase the potential for using such cells as model systems for the study of haemopoiesis. For example, it has been shown that the use of both IL-3 and SCF results in the extensive proliferation of undifferentiated myeloid blast cells purified from fetal liver and that a subsequent change in the culture medium to a high concentration of G-CSF and to low concentrations of IL-3 results in the cultures generating granulocytes alone (C Bunce, Personal Communication). Such a physiological model system for the study of human granulopoiesis is a significant advance and it is likely that further manipulations of the purified cells will generate additional knowledge of considerable value.

In addition to their use in model systems to study events in haemopoiesis fetal liver cells have been investigated as a source of pluripotent haemopoietic stem cells for experimental and clinical allogeneic transplantation. The stimulus for this research has been a search for sources of PHSCs with a decreased capacity to induce graft-versus-host-disease (GVHD). The fetal liver is the prominent site of haemopoiesis in mid-gestation and contains large numbers of PHSCs but, unlike adult bone marrow or neonatal cord blood, the fetal liver contains few if any immunocompetent lymphocytes and is therefore associated with a decreased risk of inducing GVHD (Chaplin and Gale, 1980). However, fetal liver transplants in this regard may resemble adult bone marrow transplants depleted of T cells which are associated with an increased risk of graft failure and leukaemia relapse.

Early studies in rodents indicated that fetal liver transplantation using histocompatible donors readily results in the successful haemopoietic engraftment and immune reconstitution of irradiated recipients without the induction of GVHD (Uphoff, 1958). Further studies, including those in other mammal model systems, suggested that successful fetal liver transplantation could also be achieved across major histocompatibility barriers but that this required larger numbers of donor cells and more intensive pre-transplant conditioning of the host. It was also noted that older fetal livers provided more cells and were associated with a higher rate of engraftment but also with more GVHD (Rogo et al, 1987).

However, studies using dogs have indicated that both host conditioning and matching for major histocompatibility antigens are crucial factors required for the prevention of rejection of fetal liver grafts (Champlin et al, 1985; Primmer et al, 1985).

These experimental findings are consistent with the results which have been observed with the empirical use of fetal liver grafts in man. Most such transplants have been unsuccessful due to graft failure resulting from a combination of factors including the use of mismatched grafts, inadequate numbers of transplanted cells and inadequate pre-transplant conditioning regimes (Gale, 1987). However successful transplantation using mismatched fetal liver cells has been achieved in recipients with

severe combined immunodeficiency (SCID) where graft rejection is not a major problem and where pre-transplant immunosuppression is not required (Gale et al, 1987).

Fetal liver transplantation may have a future in the areas of immunodeficiency, aplastic anaemia and inherited and metabolic disorders if the problems of graft rejection can be overcome. A role for the treatment of leukaemias would appear unlikely as the beneficial effect associated with allogeneic bone marrow transplantation (the graft-versus-leukaemia effect) typically occurs in the context of GVHD, and is therefore less likely to develop to a significant degree following fetal liver transplantation. The data also suggest that full matching for major histocompatibility antigens may be required and that recipients will require adequate pre-transplant immunosuppressive conditioning regimens. However, the recent improvements in cryopreservation techniques and methods for histocompatibility testing permit such procedures to be undertaken and furthermore, the recent availability of cytokines which expand early haemopoietic precursor cells in vitro, such as stem cell factor (Brandt, et al, 1990; Srour et al, 1991; Brandt et al, 1992; Srour et al, 1993; Brugger et al, 1993; Muench et al, 1993), may enhance the engraftment capability of such donor cells. Such transplants indeed may ultimately prove superior to current attempts using bone marrow donations, depleted of T cells, from unrelated individuals matched for the major histocompatibility loci.

A recent imaginative use of fetal liver cells has been their transplantation in utero. Fetal tolerance, which leads to permanent chimerism, occurs naturally in non-identical animal and human twins which share a placental circulation. This observation suggests that the fetus is both an ideal donor and recipient of PHSCs as no graft rejection or GVHD occurs under these conditions. Such a strategy, which would not require matching for histocompatibility antigens, could be used for the treatment of major genetic disorders, such as haemoglobinopathies, which could be diagnosed and treated antenatally. Such experimental in utero transplants of fetal liver haemopoietic stem cells have already been carried out successfully in rhesus monkeys (Harrison et al, 1989) and three such recent transplants performed in humans have also been

partially successful (Touraine, 1992). Furthermore, the recent demonstration of successful transplantation of human PHSCs in utero into sheep fetuses (Srour et al, 1992) has provided more evidence of the potential of this therapy and the further development of human in utero transplantation is eagerly awaited.

Neonatal umbilical cord blood has also been proposed as an alternative source of PHSCs for allogeneic transplantation and indeed such cells have been used to successfully transplant HLA-identical siblings with Fanconi's anaemia (Broxmeyer et al, 1989; Gluckman et al, 1989). However these individual cases have been highly unusual, in being HLA-identical sibling transplants and the technique suffers from other disadvantages which are likely to limit its broader application. For example, in addition to the limited numbers of cells available for infusion, such grafts are likely to be associated with a greatly increased risk of GVHD due to the presence of mature T cells of both donor and maternal origin (Linch and Brent, 1989; Nathan, 1989). However, this form of transplantation is also the subject of ongoing investigation.

CHAPTER 18. STUDIES ON A CASE OF PRIMARY MYELOFIBROSIS

This study also included investigation of an individual case of primary myelofibrosis. The patient's peripheral blood mononuclear cells (PBMNC) were initially examined for their expression of surface antigens and to enumerate colony forming cells present in order to assess whether such cells might be profitably used as a source of haemopoietic precursor cells for experiments on haemopoiesis. The surface antigens expressed by these cells (p 169) indicated that, although primitive cells were present, they represented only a small minority of the total population which contained large percentages of mature cell types e.g. granulocytes, monocytes, T cells and B cells. Therefore to obtain these low frequency cells by negative selection would require a more complex antibody cocktail than that successfully used in the studies on human fetal liver. However, the purification of haemopoietic precursor cells from PBMNC of patients with primary myelofibrosis is worthy of further consideration in the longer term.

Analysis of the colony forming ability of PBMNC from the above patient consistently showed greatly increased numbers of CFU-GM which is commonly observed in primary myelofibrosis but a complete absence of circulating erythroid progenitors which are only rarely observed in these patients. For example studies of circulating erythroid progenitors in myelofibrosis patients have showed normal or increased levels in 18/18 cases studied by Carlo-Stella et al, 1987, 17/18 cases studied by Partenen et al, 1982 and 2/2 cases studied by Douer et al, 1983. However, other cases of PMF have been reported in which erythroid progenitor cells were absent. In the study of Hibbin and co-workers erythroid progenitors were absent in four splenectomised patients (Hibbin et al, 1984) and Croizat et al, 1983 observed an absence of erythroid progenitors in four patients two of which had been splenectomised. The absence of circulating erythroid progenitors in PMF patients is not invariably linked to splenectomy since the patient reported in this study and the one identified by Partenen and co-workers with absent BFU-E (Partenen et al, 1982) were not splenectomised. Furthermore, the two larger studies of 18 patients showed normal or

increased levels of circulating BFU-E in many splenectomised patients (Partenen et al, 1982; Carlo-Stella et al, 1987).

The absence of circulating erythroid progenitors in this case suggested a basic defect in erythropoiesis which was supported by other clinical, morphological and experimental features. The morphological features of the trephine biopsy indicated that the patient belonged to an uncommon but distinct subgroup of patients with PMF and red cell aplasia (Barosi et al, 1983) and the subsequent clinical course of blast transformation was entirely consistent with that previously reported for patients with this particular condition (Bentley et al, 1977; Barosi et al, 1983). Previous observations have suggested that circulating committed progenitors in PMF arise largely from the spleen (Douay et al, 1987; Craig et al, 1991) and, therefore, an alternative explanation for the failure to detect circulating erythroid progenitors in this case may be due to the fact that they were generated and sequestered in the spleen.

In this case an intrinsic defect in erythropoiesis due either to a defect in the capacity of haemopoietic stem cells to undergo commitment to the erythroid lineage or the ability of committed erythroid progenitors to undergo erythropoiesis, is more likely for the following reasons. Douay and co-workers have shown that in PMF patients, CFU-GM can be maintained in liquid suspension culture in the absence of a substantial stromal layer over a 10 week period and concluded that primitive stem cells circulated in PMF patients (Douay et al, 1987). In our study, PBMNC were cultured with normal marrow stroma to maintain circulating primitive stem cells and to assess whether they were able to give rise to erythroid progenitors in an appropriate microenvironment. Erythroid progenitor cells, were not generated in these experiments but the presence of circulating stem cells in the patient's blood was demonstrated by the generation of increased numbers of CFU-GM following incubation with marrow stroma as compared with control PMF PBMNC cultured in the absence of stroma. The failure to detect circulating erythroid progenitors for technical reasons could be excluded since the assays were controlled by the demonstration of erythroid progenitors in appropriate numbers of both normal fetal liver and bone marrow cells.

Of those PMF patients previously studied with absent circulating erythroid progenitors, cytogenetic data is available for only one other case which exhibited a complex karyotype - 47, XX, 5q-,11q-,-20, +mar 1, + mar 2 (Partenen et al, 1982). In the case reported in this thesis, a previously unreported complex karyotype was recorded (p 171) involving a translocation between chromosome 2 and chromosome 11 to (2;11)(q24/31;q13). However, a common abnormality involving the long arm of chromosome 11 was present in both the previous case and the one studied. This may suggest a causal relationship between an abnormality on the long arm of chromosome 11 and defective erythropoiesis. The breakpoint observed in the case studied here was 11q 13 [t(2;11)(q24/31;q13)] and an abnormality at 11q13 has been reported once previously in PMF but whether erythropoiesis was defective in this case is unknown (Sessarego et al, 1983). A review of the literature with regard to oncogene associations at the relevant breakpoints in the case described in this thesis revealed that proto-oncogene SEA (S13 avian erythroblastosis oncogene homologue) maps to the 11q13 region (Williams et al, 1987; Nordenskjold et al, 1989; Hayman et al, 1985). As this, therefore, may have highlighted a key gene in the regulation of erythropoiesis, which may have been altered in this patient, it was of interest to explore its involvement at the molecular level. However, subsequent DNA analysis using a probe for pSEA (p 176) indicated that this locus had not been disrupted by the (2;11) translocation. Other candidate genes for involvement in this patient's disease include - a virus integration site, int 2 which, is also located in the region of the 11q13 breakpoint and the gene for glycophorin C and a homeobox gene are both located in the region of the breakpoint on chromosome 2.

Cytogenetic analysis of additional patients with an absence of circulating erythroid progenitors is worthy of further study. This approach may reveal a common chromosome abnormality and point to the location of genes which encode key intrinsic regulators of erythroid lineage development. Furthermore, studies of the lesions in the generation of other various committed progenitor cells in PMF patients in relation to possible karyotypic abnormalities offers a useful approach to

the analysis of the chromosomal location and organisation of genes which control the generation and differentiation of haemopoietic progenitor cells.

Of additional interest in this case was the documentation of karyotypic abnormalities in pooled GM colonies isolated from the patient, the attempt to demonstrate the cytogenetic abnormalities in EBV-transformed lymphocytes and the co-culture experiments of PMF PBMNC with irradiated allogeneic marrow stroma. Cytogenetic abnormalities have been reported previously in pluripotent haemopoietic progenitor cells isolated from patients with PMF (Ruutu et al, 1983; Sato et al, 1986; Sugiyama et al, 1989). Along with the finding of G-6-PD isoenzyme restriction in patients' peripheral blood cells but not in fibroblasts, this strongly supports the hypothesis that the primary defect in PMF is a clonal expansion of pluripotent haemopoietic progenitor cells associated with reactive marrow fibrosis (Jacobson et al, 1978).

The observation that increased numbers of CFU-GM were generated following incubation of PMF PBMNC with normal marrow stroma as compared with control PMF PBMNC cultured in the absence of stroma supports the concept that pluripotent haemopoietic stem cells may circulate in PMF patients. This concept was originally suggested by Douay et al in 1987, who observed that CFU-GM obtained from PMF PBMNC can be maintained in liquid suspension culture in the absence of a substantial stromal layer over a 10 week period. To test this hypothesis further Epstein-Barr virus transformed B cell lines were generated from the patient with PMF and then subjected to karyotypic analysis to determine whether any cells of the B lineage carried the same karyotype abnormality as the patients myeloid progenitor cells. Twenty lymphoblastoid cell lines were derived and cloned from B cells from this patient but cells from all the lines exhibited a normal male karyotype. This observation suggests that either 1) the clonal disorder giving rise to myelofibrosis did not involve B lineage precursors in this patient or 2) the lymphoblastoid cell lines had been generated from long lived B cells that had themselves been generated from normal B cell progenitors. However this approach, which has not been used before, is worthy of further study in other patients with PMF.

In conclusion the central problem in understanding haemopoiesis is now the endeavour to describe the events within developing blood cells which regulate the complex and integrated processes of their proliferation, lineage commitment and maturation. As outlined in this study many of the microenvironmental factors which regulate these processes have been identified. In particular many haemopoietic growth and/or differentiation factors have been molecularly cloned and are now available as research tools and, increasingly, as potential therapeutic agents. Furthermore, in parallel to our improved knowledge of the cytokines and microenvironmental factors which regulate haemopoiesis, it is becoming possible to recognise, characterise and purify haemopoietic progenitor cells in both mouse and man.

Taken together, these advances make it possible to execute key in vitro culture experiments designed to shed light on intracellular events related to haemopoiesis. The studies outlined in this thesis highlight this concept and show how studies of both normal primitive myeloid cells and those from patients with diseases such as PMF can be used to seek out and study the genes and metabolic pathways which are involved in the regulation of lineage commitment, proliferation and differentiation with haemopoietic cells. As yet it is not possible to undertake detailed studies on pluripotent haemopoietic stem cells, but as I hope this thesis has illustrated, the field is making rapid progress towards this goal.

APPENDIX A BIBLIOGRAPHY

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APPENDIX B. PUBLICATIONS RELATED TO THESIS

(i) Patton W N, Bunce C M. Scott S A. Lord J M, Brown G (1990). Phorbol myristate acetate induction of monocyte differentiation of normal myeloid blast cells isolated from human fetal liver. Br.J.Haematol. 74 (Suppl.1): 41(abstract).

(ii) Bunce C M, Patton W N, Pound J D, Lord J M, Brown G (1990). Phorbol myristate acetate treatment of normal human myeloid blast cells promotes monopoiesis and inhibits granulopoiesis. Leukaemia Research 14: 1007-1017.

(iii) Patton W N, Bunce C M, Larkins S, Brown G (1991). Defective erythropoiesis in primary myelofibrosis associated with a chromosome 11 abnormality. Br.J.Cancer 64: 128-131.

(iv) Patton W N, Bunce C M. Larkins S, Brown G (1991). Defective erythropoiesis in myelodysplastic syndromes. J.Clin.Pathol. 44: 965 (correspondence).

(v) Bunce C M, French P J, Patton W N, Turnell A S, Scott S A, Michell R H, Kirk C J, Brown G (1992). Levels of inositol metabolites within normal myeloid blast cells and changes during their differentiation towards monocytes. Proc. R . Soc.Lond. B 247: 27-33.

PHORBOL MYRISTATE ACETATE INDUCTION OF MONOCYTE DIFFERENTIATION OF NORMAL MYELOID BLAST CELLS ISOLATED FROM HUMAN FOETAL LIVER. Patton WN, Bunce CM, Scott SA, Lord JM, Brown G. Department of Immunology, Medical School, Birmingham, B15 2TJ.

The molecular events associated with the proliferation, lineage committment and differentiation of normal haemopoietic cells are relevant to the perturbations involved in leukaemias and myelodysplastic syndromes. However, studies of these events have been difficult to perform as they require the purification of homogeneous haemopoietic precursors in large numbers for the relevant biochemical and molecular analyses. We have purified an undifferentiated myeloid blast cell population from 16-20 wk human foetal liver. The procedure involves ficoll gradient fractionation of the initial cell suspension and subsequent removal of contaminating erythroblasts and macrophages by indirect erythrocyte rosette sedimentation using monoclonal antibodies directed against glycophorin and a macrophage associated surface antigen. Total yields of $7-9x10^6$ cells were obtained depending on the age and size of the liver. The purified population consisted mainly of undifferentiated blast cells(70-80%) which where negative for Sudan Black B, chloroacetate esterase, a-naphthyl acetate esterase(ANAE) and markers of mature B and T cells. During 3 weeks in culture the cells spontaneously differentiated into both mature granulocytes (as identified by morphology and expression of lactoferrin and a granulocyte associated antigen) and macrophages (as identified by morphology and ANAE expression). When cultures were treated with 10nM phorbol myristate acetate, terminal monocyte differentiation occurred within 48hr in 60% of the blast cell population. The purified blast cells offer a model system for the study of molecular and other events associated with normal myeloid and particularly monocyte maturation.

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PHORBOL MYRISTATE ACETATE TREATMENT OF NORMAL HUMAN MYELOID BLAST CELLS PROMOTES MONOPOIESIS AND INHIBITS GRANULOPOIESIS

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Abstract—Fractionation of mononuclear cells from human fetal liver provides a cell population at early stages of myeloid differentiation which, when cultured, generates neutrophils and macrophages for up to a month. These studies describe the further purification of an undifferentiated myeloid blast cell population by rosette sedimentation of unwanted cells, after coating these cells with monoclonal antibodies which identify macrophages and erythroblasts. In culture, the purified blast cells generated only neutrophils and macrophages. When treated with 10 nM PMA, 62% of the purified cells were induced to differentiate towards macrophages within 48 h. PMA-induced cells acquired morphological features of macrophages and synthesized α-naphthyl acetate esterase. The differentiation of the remaining blast cells towards neutrophils, seen in untreated cultures, was completely inhibited by PMA, as revealed by the absence of increases in the numbers of cells expressing lactoferrin and an antigen which appears at the promyelocyte stage of differentiation. Thus, PMA effects intracellular changes which both promote monopoiesis and inhibit granulopoiesis, suggesting a reciprocal interaction between intracellular processes which regulate the capacity for the two pathways of maturation. The purified blast cell population provides a good model system for studies of molecular events which regulate the expression of macrophage characteristics.

Key words: Myelopoiesis, phorbol esters, macrophages, fetal liver.

INTRODUCTION

STUDIES of the cellular events during myeloid cell differentiation have most often involved the use of cell lines as model systems. However, human promyeloid cell lines such as HL60 [1], KG-1 [2] and ML-1 [3] have all been derived from leukaemia cells and present abnormal karyotypes. Therefore, there is a difficulty as to whether the cellular processes described from studies of transformed myeloid cell lines reflect either events during normal myelopoiesis or some aspect of the lines' abnormal status. To confirm that intracellular changes defined in studies of myeloid cell lines are a feature of normal myelopoiesis and also to delineate events pertinent to malignant transformation, it is essential to purify myeloid cells at a very early stage of differentiation in sufficient numbers for biochemical and molecular biological studies.

In a previous study, we used a one-step fractionation procedure to obtain a mononuclear cell population from human fetal liver which was capable of generating all stages of neutrophil and macrophage differentiation [4]. This cell population provided a good source of myeloid precursor cells but was contaminated with erythroblasts and mature macrophages. In this study, we have further purified an undifferentiated myeloid blast cell population by rosette sedimentation of the above contaminating cell populations. The myeloid blast cell population was purified by negative rather than positive selection so as to avoid interference with surface properties which may elicit events which, in turn, confuse subsequent physiological studies. The purified myeloid blast cells are described in terms of their morphology, phenotype and capacity to differentiate towards neutrophils and monocytes. In particular, the effect on this purified myeloid population of PMA, which is used to induce HL60 cells to differentiate rapidly towards monocytes [5], was studied in detail.

Abbreviations: PMA, phorbol myristate acetate; *FCS*, fetal calf serum; *BSA*, bovine serum albumin; *GM-CFC*, granulocyte/macrophage colony forming cell; *PHA-LCM*, phytohaemagglutinin stimulated leucocyte conditioned medium; *CSF*, colony stimulating factor; *^{3}[H]TdR*, tritiated thymidine.

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MATERIALS AND METHODS

Purification of blast cells

Light-density cells were harvested from whole human fetal liver as previously described [4]. Normoblasts and macrophages were removed by an indirect rosetting technique [6] using mouse monoclonal antibodies against glycophorin C, Ret 40F (Dr D. Y. Mason, Oxford, [7]) and a monocyte specific surface antigen, 61D3 [8]. Cells were resuspended in 500 μl aliquots at 4×10^6/ml and incubated with antibody to glycophorin C (500 μl of undiluted tissue culture supernatant) and 61D3 antibody (20 μl of undiluted ascitic fluid) for 15 min at room temperature. Following two washes with RPMI 1640 medium (GIBCO, Paisley) supplemented with 2% FCS (GIBCO), 250 μl of sheep erythrocytes coated with anti-mouse immunoglobin [9] were added and the cells centrifuged at 300 ***g*** for 3 min. After a final 5 min incubation at 4°C the cells were resuspended and pooled. An aliquot was taken for enumeration of rosette positive cells. The remaining cells were made up to 6 ml with RPMI 1640 medium supplemented with 2% FCS, the rosette positive cells and excess sheep erythrocytes were removed by further fractionation on 3 ml of Ficoll–Hypaque (Pharmacia, Milton Keynes). The light-density cells were harvested and washed three times with RPMI 1640 containing 5% FCS. Aliquots were removed to provide cytospin preparations (Cytospin 2, Shandon Scientific Ltd, Runcorn) for Romanowsky, cytochemical and immunostaining, for assessment of phagocytosis and indirect rosette assays for glycophorin C and 61D3 positive cells. The rosette assays were performed as described above.

For culture, cells were aliquoted, in 1 ml volumes at 2.5×10^5/ml into individual wells of 24-well tissue culture plates (Nunc Ltd, Paisley) in RPMI 1640 medium supplemented with 20% heat inactivated FCS (GIBCO), 10 μM hydrocortisone sodium succinate (Organon, London), 5 μg/ml vitamin D_3 (cholecalciferol, Sigma, Poole) and 100 U/ml penicillin–50 μg/ml streptomycin (GIBCO). PMA-treated wells contained 10 nM PMA (Sigma). Individual wells of untreated and PMA-treated cells were harvested after 16, 42 and 66 h for assessment of cell number, viability, numbers of phagocytic cells and cytospin preparation for subsequent Romanowsky, cytochemical and immunocytochemical analyses.

Assessment of colony forming ability

The number of cells within the purified blast cell population able to form colonies in semi-solid medium was determined by using a GM-CFC assay similar to the method described by Ash *et al.* [10]. Briefly, 5×10^4 purified fetal liver cells were seeded in triplicate in a 1 ml volume of Iscove's Modified Dulbecco's Medium (GIBCO) containing 1.2% methylcellulose (Sigma), 20% FCS, 1% deionized BSA (Sigma), 5×10^{-5} M 2-mercaptoethanol (Sigma) and 5% PHA-LCM as a source of growth factors. Colonies containing ≥40 cells were enumerated at day 12 and the experiment was performed in duplicate.

Assessment of DNA synthesis

DNA synthesis was assessed by pulsing cultures of purified blast cells in flat-bottomed 96-well microtitre plates. Cells were seeded at 10^5 per well in 100 μl of the above medium, PMA-treated wells contained 10 nm PMA. Half a μCi of ^{3}[H]TdR (25 Ci/mM) was added in 50 μl of medium at 0, 12, 36, 60 and 84 h and cultures were harvested 6 h later on a Skatron automatic harvester. ^{3}[H]TdR incorporation was measured by scintillation counting. Incorporation determinations were performed in triplicate and the experiment was undertaken twice.

Assessment of differentiation

In all analyses a minimum of 400 cells were assessed. Differential cell counts were performed by light microscopy following staining of cytospin preparations with May–Grunwald–Giesma. Staining for monocyte specific esterase was performed using α-naphthyl acetate as the substrate [11]. Granulocyte differentiation was assessed by staining cells with the monoclonal antibody AGF4.48, which recognizes cells at the promyelocyte to neutrophil stages of maturation [12], and by immunocytochemical staining for lactoferrin, a consistuent of neutrophil secondary granules [13]. AGF4.48 staining was by indirect immunofluorescence. Unfixed cytospin preparations were incubated with AGF4.48 tissue culture supernatant for 30 min at room temperature, followed by a fluorescein conjugated sheep anti-mouse immunoglobulin, diluted 1 in 50 in a mixture of 10% normal sheep serum and 5% FCS. Lactoferrin staining was performed by an indirect immunoperoxidase technique. Fixation and blocking of endogenous peroxidase activity was achieved by incubation for 20 min in methanol–formalin (1:1) containing 0.6% hydrogen peroxide. After washing, incubation for 20 min at room temperature with a mixture of 20% normal human AB serum and 20% swine serum was used to prevent non-specific binding. Cytospin preparations were then incubated for 30 min at room temperature with a 1 in 50 dilution of a polyclonal sheep IgG antibody to lactoferrin (PC062, The Binding Site Ltd, Birmingham) and for a further 30 min with horseradish peroxidase conjugated swine anti-sheep IgG (PP361, The Binding Site Ltd) at a 1 in 100 dilution. Following colour development with 3,3′-diaminobenzidine, the nuclei were counterstained with Mayer's Haemalum. Phosphate buffered saline, pH 7.1 containing 0.1% BSA (Sigma) was used as the diluting and washing buffer for the above two staining procedures.

Immunocytochemical staining for mature T and B lymphocytes was performed by the immunoalkaline phosphatase technique using monoclonal antibodies to the CD3 and CD22 antigens as previously described [14]. To prevent non-specific binding, 0.1% BSA was added to the wash and dilution buffers and dilutions of the primary and second antibody were supplemented with 10% normal rabbit serum. The CD3 antibody (M756, diluted 1 in 100), CD22 antibody (M738, diluted 1 in 200), rabbit anti-mouse immunoglobulin (Z259, diluted 1 in 25) and alkaline phosphatase–anti-alkaline phosphatase complexes (D651, diluted 1 in 50) were purchased from Dako Ltd (High Wycombe). In all immunostaining procedures positive, negative and substitution controls were included.

Phagocytic function was assessed by the ability of cells to phagocytose complement-coated yeast cells as previously described [15].

RESULTS

Purification of myeloid blast cells

A one-step Ficoll–Hypaque density separation of human fetal liver cells provided light-density cells consisting of similar percentages of blasts (46%) and normoblasts (29%). The remaining 25% of the cells were

TABLE 1. PURIFICATION OF UNDIFFERENTIATED BLAST CELLS FROM FETAL LIVER

Characteristic	Pre-rosette separation (%)*	Post-rosette separation (%)*
Differential:		
Normoblasts	29 ± 3.2	<1
Blasts	46 ± 6.0	73 ± 4.0
Promyleocytes/myelocytes/metamyelocytes	4 ± 1.5	8 ± 3.2
Neutrophils	1 ± 0.3	1 ± 0.4
Promonocytes	2 ± 0.4	3 ± 1.4
Macrophages	4 ± 1.4	<1
Lymphocytes†	2 ± 0.4	4 ± 1.1
Others	12 ± 1.9	11 ± 2.2
61D3/Ret 40F positive cells‡	33 ± 0.6	4 ± 0.1
Yield	—	25 ± 2.1

* Data are the mean ± S.E. of values obtained from four separate experiments.
† Combined percentages of CD3 (T) and CD22 (B) positive cells.
‡ Determined by indirect rosetting.

mostly macrophages (4%), cells at the promyelocyte to neutrophil stages of maturation (5%), lymphocytes (2%), as identified by the expression of the CD3 and CD22 antigens, and cells which were not readily identifiable and were classed as others (12%). The growth and differentiation of these cells in liquid suspension culture has already been described in detail [4]. Proliferation was maintained for up to a month and during this time the number of normoblasts declined rapidly and mature neutrophils and macrophages appeared in culture. By day 8, neutrophils and macrophages each accounted for 10% of the viable leucocytes and, by day 24, 25% and 30% of these cells were neutrophils and macrophages, respectively.

Normoblasts and macrophages were removed from the light-density fetal liver cells using Ficoll–Hypaque to separate rosetting cells, prepared after labelling the above cells with monoclonal antibodies which identify glycophorin C (Ret 40F) and a monocyte-associated antigen (61D3), respectively. The percentage of cells rosetting when a combination of the two antibodies was used (33%) agree with the combined percentages of normoblasts and macrophages as identified by their morphology (33%). Table 1 shows the differential obtained for the Ficoll–Hypaque interface population after sedimentation of rosettes. Normoblasts and macrophages were successfully removed as shown by the percentages of these cells observed in Romanowsky stained preparations (<1%) and the combined percentages of cells rosetting after relabelling with the monoclonal antibodies Ret 40F and 61D3 (4%). The purified cell population consisted mostly of undifferentiated blast cells (73%) which were pleomorphic exhibiting variation in cell size and both nuclear and cytoplasmic characteristics (see Fig. 2). The chromatin pattern was open with multiple nucleoli and the nuclear outline varied from regular and round to mainly irregular convoluted forms. The cytoplasm was either scanty or moderate in quantity, weak to strongly basophilic and often vacuolated. Azurophilic granulation was absent. The remaining contaminating cells were at the promyelocyte to neutrophil stages of differentiation (9%), lymphocytes (4%) and unidentified cells (11%). A cell yield of 25% was routinely obtained, providing 7–9 × 10^6 cells from each fetal liver, depending on the age and size of the liver.

When the purified blast cells were assayed for their ability to form colonies in semi-solid medium, containing PHA-LCM as a source of CSF, the number of colonies observed in two experiments was 234 and 264 per 10^5 cells. The colonies consisted of granulocytes and/or macrophages and their precursors as revealed by staining of cytocentrifuged preparations of single colony contents. The initial frequency of GM-CFC in the light-density cells from fetal liver in this and the previous study [4] was between 100 and 200 GM-CFC per 10^5 cells. Thus, significant enrichment of GM-CFC was not observed within the purified blast cell population and the majority of these cells do not form colonies.

Promotion of macrophage differentiation within purified blast cells

The myeloid cell population obtained from fetal liver has been shown previously to expand into all stages of differentiation during one month in culture [4]. However, the maturation is gradual such that mature macrophages are only present at appreciable

levels (10% of cells) by day 10. Similarly, the purified blast cells gradually matured when cultured and after six days only 12% of cells expressed the enzyme α-naphthyl acetate esterase, a marker for macrophages. Thus, the further purification of the myeloid blast cells made no difference to the generation *per se* of mature macrophages and also granulocytes. As in the case of the light-density mononuclear cell fraction initially described from fetal liver [4], granulocyte maturation occurred in cultures of purified blast cells such that after six days 41% of cells were at the promyelocyte to neutrophil stages of maturation. The possibility that the purified blast cell population comprises to any appreciable degree of primitive erythroblasts is excluded by the observation that the blast cells survived well in culture. By 48 h, 51% of cells had retained a blast cell morphology giving rise to a value for the total number of blast cells maintained of 58% of the initial population isolated. The reduction in blast cell numbers can be accounted for by cells gradually progressing to later stages of differentiation. In contrast, normoblasts and erythroblasts present in the unpurified light-density population from fetal liver die very rapidly in culture [4].

PMA was added immediately after establishing cultures of purified blast cells at 2.5×10^5/ml. In both the PMA-treated and control cultures there was no increase in the total number of cells over the 66-h period during which analyses were undertaken and values obtained for the number of viable cells recovered (80–92%) and cell viability (87–98%) were similar for the treated and untreated cultures throughout the entire course of each experiment. Assessment of cell proliferation by analysis of DNA synthesis revealed that cells within the PMA-treated cultures had proliferated to a lesser extent than those in control cultures. In control cultures there was a moderate increase in ^{3}[H]TdR uptake. Values for dpm incorporated per 10^5 cells were 3.0×10^4 at time 12 h rising to 5.1×10^4 and 4.2×10^4 at 60 and 84 h, respectively. At 12 h PMA-treated cultures showed an incorporation of 1.7×10^4 dpm per 10^5 cells rising to values of 4.2×10^4 and 3.4×10^4 per 10^5 cells at 60 and 84 h, respectively. Thus, it is unlikely that PMA had induced rapid and extensive proliferation of a minor population of the purified blast cells and that this together with loss of a considerable number of cells within another cell population had maintained cell numbers at a constant level in the PMA-treated cultures. Furthermore, large numbers of dead cells were clearly not present in the wells.

Twenty-four hours after the addition of PMA, the morphological appearance of cells in the treated cultures was already different from that of cells in control cultures. A considerable proportion of the cells in PMA-treated cultures had become pleomorphic, spread, adherent and produced pseudopodia. Cells with these features, which are consistent with cells having undergone macrophage differentiation, were less evident in the control cultures.

To what extent the blast cells had rapidly differentiated towards macrophages, was assessed by determining the percentage of cells classed as promonocytes and macrophages in Romanowsky stained preparations, those expressing α-naphthyl acetate esterase and the capacity of cells to phagocytose complement coated yeasts. The cells enumerated as promonocytes in stained preparations had distinguishing characteristics of increased size with increased quantities of cytoplasm which was less basophilic, vacuolated and had irregular edges. Phagocytosis of a few contaminating sheep erythrocytes, from the rosette procedure, was also occasionally evident. The nuclear to cytoplasm ratio was reduced with an eccentrically placed nucleus which was reniform in shape and contained nucleoli. More mature forms showed typical macrophage morphology. At time zero, cells with these features were present at the level of <1%. As shown in Fig. 1 and Fig. 2, the addition of 10 nM PMA resulted in an increase in the percentage of cells classed as promonocytes and macrophages to values of 62% and 65% at 42 h and 66 h, respectively, compared with values of 14% and 16% in control cultures. The rapid morphological differentiation towards macrophages seen with PMA was accompanied by an increase in the number of cells expressing α-naphthyl acetate esterase. Four percent of the initial cell population expressed this enzyme. By 66 h, 46% of cells in PMA treated cultures were positive as compared with 8% of control cells (see Figs 1 and 2). The percentage of cells expressing α-naphthyl acetate esterase, together with no increase in cell numbers over 66 h and a high recovery of cells from wells, reveals an increase in the total number of mature macrophages from 1.0×10^4 at time zero to 9.7×10^4 in PMA-treated cultures at 66 h as compared with 1.8×10^4 in control cultures at this time point. The number of phagocytic cells, which includes both mature macrophages and neutrophils, increased from <1% to 17% at 66 h in PMA-treated cultures as opposed to 6% in control cultures.

Effect of PMA on granulopoiesis

In PMA-treated cultures the maximum percentage of cells which could be induced to differentiate towards macrophages was 63–67%. At 66 h, 31–33% of the remaining cells, as observed in Romanowsky stained preparations, had retained a blast cell appearance and only 1% of total cells had differentiated

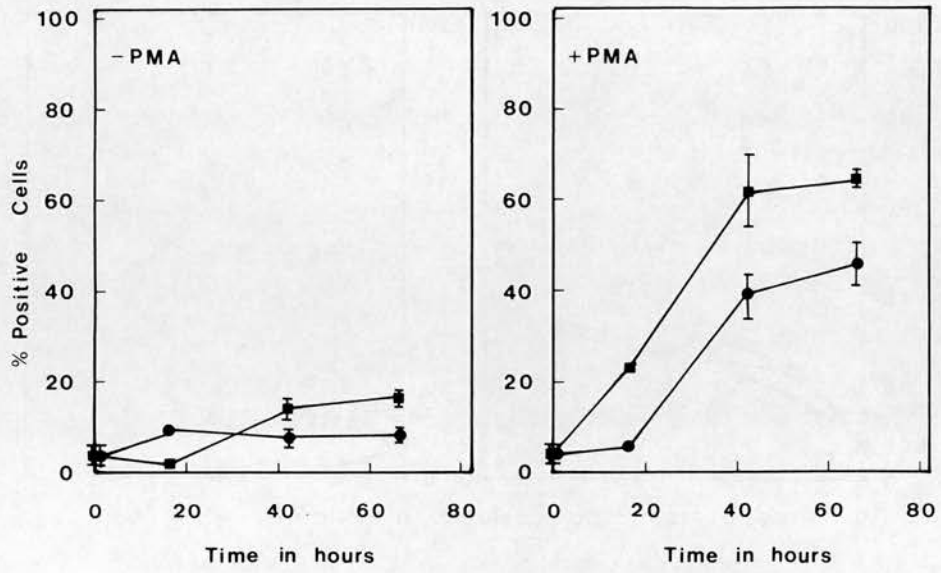

FIG. 1. Induction of monocyte differentiation by PMA. Monocyte differentiation was assessed by the percentage of promonocytes and monocytes/macrophages, as identified morphologically by Romanowsky staining, (■—■) and by the percentage of cells positive for α-naphthyl acetate esterase (●—●). Results are means ± S.E. of values obtained from four separate experiments.

towards neutrophils. In contrast, the analysis of stained preparations of cells from control cultures showed that, by 66 h, 34% of cells were at the promyelocyte to neutrophil stages of differentiation.

To confirm that the addition of PMA had interfered with granulopoiesis within the purified blast cell population, the percentage of cells expressing lactoferrin and a neutrophil cell surface antigen AGF4.48, which is expressed at the promyelocyte stage of differentiation [12], were enumerated throughout the time course of PMA-treated and control cultures. Within the starting cell population 10% of cells contained lactoferrin and expressed the AGF4.48 antigen. As shown in Fig. 3, by 66 h the numbers of lactoferrin positive and AGF4.48 antigen positive cells in control cultures had risen to 33% and 39%, respectively. Throughout the time course of the PMA-treated cultures, the percentage of lactoferrin positive cells remained at the level of 5–14% and those stained by the monoclonal antibody AGF4.48 at 9–12%. The mean of values for the percentages of AGF4.48 positive cells represent a level of 1.9×10^4 mature granulocytes in total in PMA-treated cultures as compared with 9.0×10^4 in control cultures. Initial levels of mature granulocytes, in the purified blast cell population, were in the range $1.8–2.1 \times 10^4$. Figure 2 shows that the frequency of AGF4.48 positive cells was considerably lower in cultures of PMA-treated cells than in cultures of control cells.

To exclude the possibility that the inhibition of granulopoiesis or promotion of monopoiesis observed were due to indirect effects whereby the PMA had induced the small percentage of contaminating lymphocytes (4%) to release inhibitory or inductive factors, the following experiment was undertaken. The monoclonal antibody AGF43, which stains both T and B lymphocytes [16], was added to the antibody mixture used to prepare indirect rosettes which resulted in effective depletion of lymphocytes. In the case of this experiment, light density cells prepared from fetal liver using Ficoll–Hypaque consisted of 56% blasts, 22% normoblasts, 4% macrophages and a considerable contamination by lymphocytes of 19%. Light-density fetal liver cells which had subsequently been depleted of glycophorin C positive and 61D3 positive cells comprised of 77% blasts and 21% lymphocytes. The fetal liver cell population which had been depleted of the above cells and AGF43 positive cells, was enriched in blast cells (86%) and contained only 1% lymphocytes. Parallel studies of the effect of PMA on the above two purified cell populations showed that removal of the contaminating lymphocytes did not affect the rapid induction of monocyte differentiation by PMA or inhibition of granulopoiesis. At 61 h after PMA

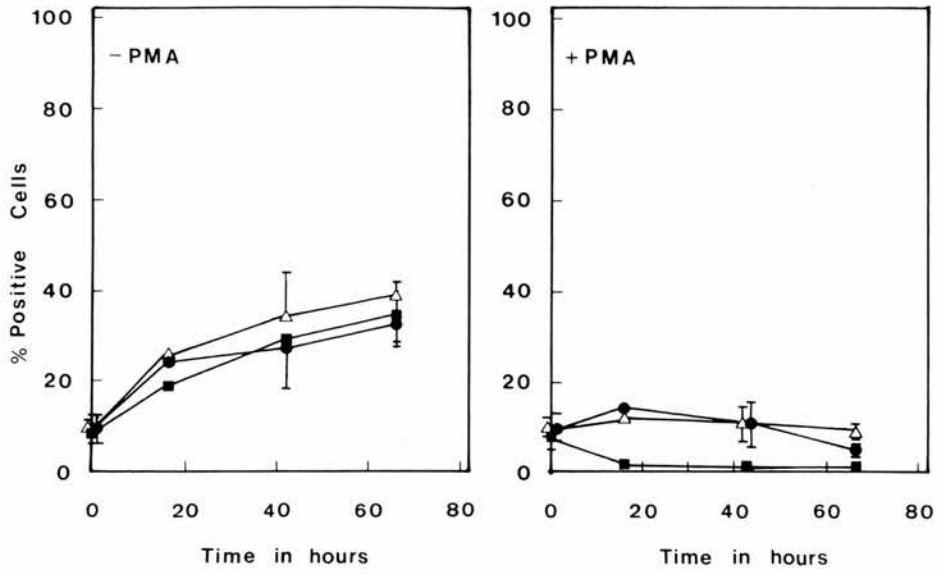

FIG. 3. Inhibition of granulocyte differentiation by PMA. Granulocyte differentiation was assessed by: the percentage of granulocyte cells (promyelocytes, myelocytes, metamyelocytes and granulocytes) as identified morphologically by Romanowsky staining (■—■); the percentage of cells positive after staining, by indirect immunofluorescence, with AGF4.48 antibody (△—△); and by the percentage of cells positive for the immunohistochemical localisation of lactoferrin (●—●). Results are means ± S.E. of values obtained from four separate experiments.

treatment, cultures established from fetal liver cells depleted of lymphocytes and from those cells contaminated by lymphocytes contained similar numbers of cells expressing α-naphthyl acetate esterase. Values obtained for the positive cells were 27% and 21%, respectively. In both cultures after treatment with PMA for 61 h there was no evidence of neutrophil differentiation as assessed by the percentages of AGF4.48 antigen positive cells (<1%) and cells at the metamyelocyte to neutrophil stages of maturation (<1%).

DISCUSSION

Negative selection techniques for enriching cell populations have the advantage that the required cell population is unperturbed and they are thus preferred over positive selection methods when cells are required for *in vitro* physiological studies. For example, B lymphocyte purification by eliminating T cells, after rosetting with sheep erythrocytes, avoids antibody labelling of the desired cells and interference with their surface properites [17]. Similarly, negative selection by panning out the unwanted cells has been recommended for the isolation of haemopoietic progenitor cells which are susceptible to non-specific damage by $CrCl_3$-coupled erythrocytes used to purify these cells by rosetting [18]. In this study we have used a simple two-step Ficoll–Hypaque procedure to purify myeloid blast cells from fetal liver so as to obtain large numbers of these normal cells for biochemical analyses. Unwanted cell populations were eliminated by rosette sedimentation after coating these cells with a mixture of two monoclonal antibodies which identify glycophorin C and a monocyte-associated antigen, respectively. Thus, the blast cells obtained were not subjected to either antibody coating or possible damage by $CrCl_3$-coupled erythrocytes as suggested previously. Furthermore, since the fetal liver cells were first purified as light-density cells using Ficoll–Hypaque, then subsequent negative selection by Ficoll–Hypaque, the fractionation depended only on the criterion of antigen expression. In contrast, when panning for negative selection after coating cells with antibody, both antigen expression and non-specific adherence are factors involved and the process may selectively remove some adherent myeloid blast cells. A minimal combination of two antibodies was used to coat unwanted cell populations, which were intensely labelled, to avoid the

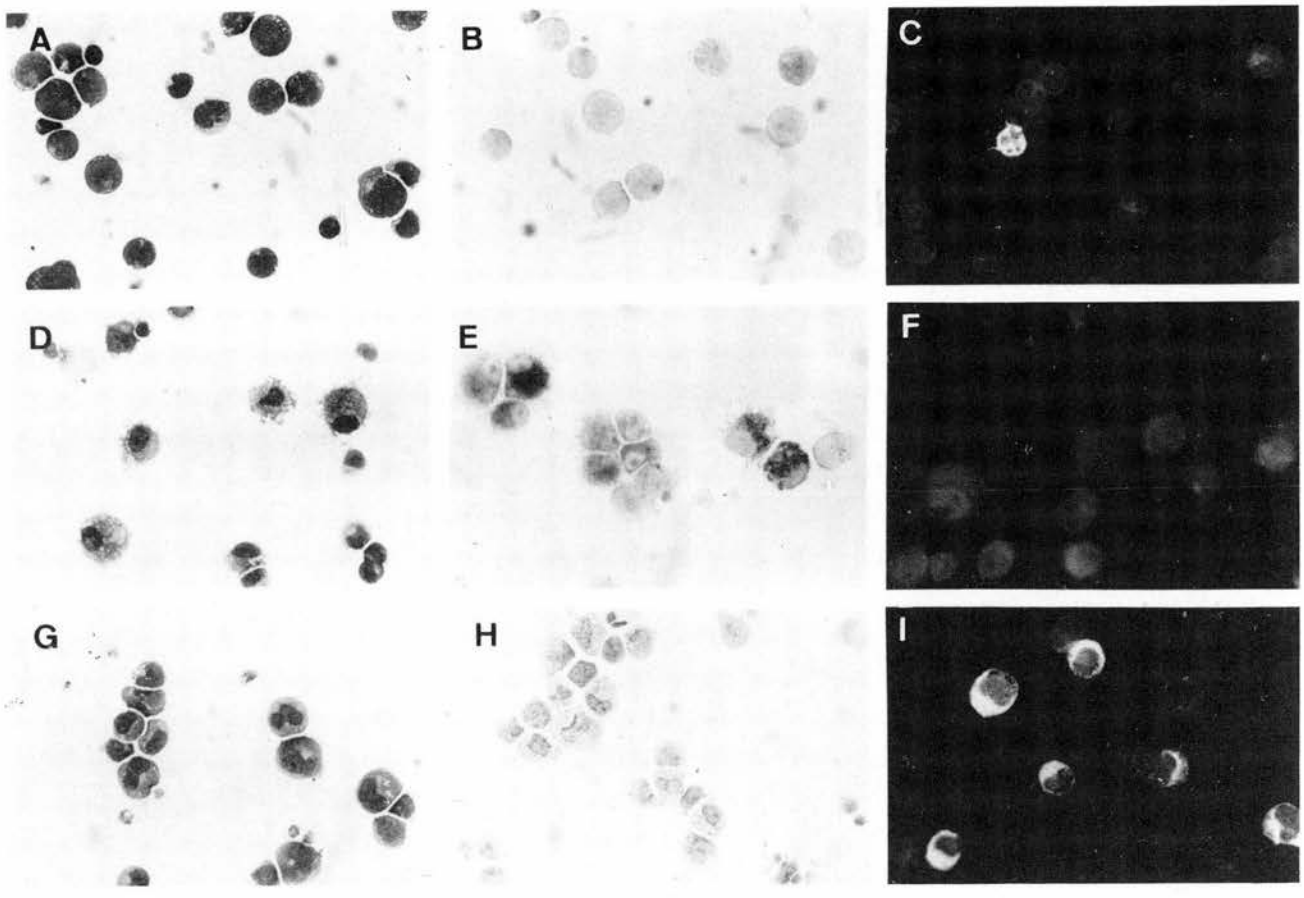

FIG. 2. PMA induction of monocyte differentiation and inhibition of granulocyte differentiation of undifferentiated blast cells from human fetal liver. Parts (A–C) show cells at 0 h; (D–F) cells treated with PMA for 66 h; (G–I) control cells at 66 h. Parts (A, D, G) are Romanowsky stained preparations; (B, E, H) are preparations stained for α-napthyl acetate esterase activity and (C, F, I) are preparations stained by indirect immunofluorescence with AGF4.48 antibody. Magnification ×500.

possibility that the combined effects of a large cocktail of monoclonal antibodies, each binding to haemopoietic cell surface antigens expressed at uncertain and/or low levels on undifferentiated myeloid cells, may generate indirect rosettes and eliminate some cells of interest.

The cell population obtained from fetal liver consisted of undifferentiated myeloid blast cells and a few contaminating lymphocytes. The majority of the purified blast cells lacked features of differentiation, such that they could not be identified as either precursors of neutrophils or monocytes. When cultured, the cells differentiated towards either neutrophils or macrophages. However, as reported previously for the light-density fetal liver cells [4], the terminal differentiation of the population as a whole occurred over a protracted time course. Both the slow change in the differentiation status of the population and that both granulocytes and macrophages are generated, presented difficulties in using these cells to analyse biochemical events pertinent to either neutrophil or macrophage differentiation.

PMA has been used to induce rapid terminal differentiation of various cell types, for example, the promyeloid cell line HL60 [5] and keratinocytes [19]. When added to cultures of purified blast cells in this study, 42–65% of the cells differentiated towards macrophages within 42 h, as revealed by increased numbers of cells with an appropriate morphological appearance and cells which expressed the enzyme α-naphthyl acetate esterase and phagocytosed yeasts. Thus, the addition of PMA provided a reduced time course for the differentiation of a population of the blast cells towards macrophages. An unexpected finding was that PMA inhibited granulopoiesis within the cultures, presumably by affecting the 31–51% of blast cells which underwent neutrophil differentiation in the control cultures. The inhibition was revealed by the lack of increased numbers of cells at or beyond the promyelocyte stage of differentiation (AGF4.48 positive) in PMA-treated cultures. The practical benefit from the rapid promotion of monopoiesis and simultaneous inhibition of granulopoiesis is that a population of normal cells is provided which is undergoing macrophage differentiation only. This is advantageous to studies of biochemical events during monopoiesis since, as suggested above, a mixture of blast cells undergoing macrophage differentiation and those differentiating towards neutrophils would be less suitable. An alternative solution to this problem would be to separate the two blast cell populations. However, appropriate markers are not presently available.

The capacity of PMA to both promote monopoiesis and inhibit granulopoiesis has important implications in relation to intracellular processes which regulate these two pathways of differentiation. The purified blast cell population is, to a large extent, a mixture of cells which are already committed to either neutrophil or macrophage differentiation, since a maximal number of 65% of the cells were induced to mature towards monocytes by PMA and, in control cultures 34% of the cells were able to spontaneously differentiate towards neutrophils. The 34% of cells able to differentiate towards neutrophils correlates well with the percentage of blast cells which did not differentiate in PMA-treated cultures (32%). Thus, PMA does not interfere with granulopoiesis by diverting cells towards the monocyte pathway of maturation. This conclusion leads to the view that the opposing effects of PMA on granulopoiesis and monopoiesis is related to a reciprocal interaction between intracellular processes which regulate the capacities for the two pathways of maturation. In other words, intracellular events effected by PMA which facilitate monopoiesis concomitantly lead to a reduced capacity for granulopoiesis. For example, PMA is known to activate protein kinase C (PKC) which may phosphorylate proteins and confer functional activity which is essential for monocyte differentiation. As irreversible commitment of progenitor cells to a particular pathway of differentiation is involved, these proteins would presumably include DNA-binding proteins, the PMA-responsive elements [20] identified as modulators of gene activity in several cell types. The reciprocal enhancement and silencing of genes required for monocyte and neutrophil differentiation respectively in this system, could thus be achieved through activation of the PKC pathway and the ultimate modulation of specific DNA-binding proteins.

The above general consideration as to the effect of PMA also accords with our previous proposal that expression of the potentials for neutrophil and macrophage differentiation is linked in the following way. Data from various studies were interpreted to suggest that the above two differentiation potentials are expressed in a progressive and linear manner, in that as cells lose the capacity for neutrophil differentiation they then acquire the potential for monocyte differentiation [21]. If this hypothesis is correct, then only one or two choices are available at any given time and lineage potentials are viewed as partially overlapping differentiation options [22]. The notion that PMA promotes monopoiesis and inhibits granulopoiesis by virtue of a reciprocal interaction between intracellular events, which regulate commitment to these two pathways of differentiation, provides a mechanistic expansion of the previous considerations. Furthermore, support for the model

proposed, that lineage potentials are sequentially expressed, is provided as follows. At the molecular level, the modulation of gene expression required for cell diversification may be effected via a relatively small group of transcriptional regulators, governed by the major intracellular signalling pathways, including protein kinase C. Specificity is introduced at the level of modulation of the activity of these gene regulators, such that they bind to their specific loci, and the accessibility of these loci, which may require the removal of repression. The minimal activation of one signalling pathway, leading to enhancement and silencing of particular genes, would necessitate the availability of only two differentiation options as regards the expression of lineage potentials. In contrast, a multipotent model of haemopoiesis in which cells can differentiate directly along any one of at least five maturation pathways [23], is likely to require the involvement of several intracellular signalling pathways, which modulate a panel of DNA-binding proteins, so as to select one differentiation option and close the others.

In conclusion, a simple procedure for purifying myeloid blast cells in considerable numbers from fetal liver is described, together with the use of PMA to promote monopoiesis whilst inhibiting granulopoiesis, providing a model system which can be used to study biochemical and genetic events in normal myeloid precursor cells undergoing monocyte differentiation. For example, changes in the phosphorylation status of proteins have been observed which relate to differentiation in HL60 cells towards monocytes [24]. Whether these proteins are phosphorylated during normal monopoiesis is an important issue. Furthermore, it would be worthwhile to screen a variety of agents, which are used to induce cell lines to differentiate towards neutrophils, to investigate whether, conversely, granulopoiesis can be promoted and monopoiesis inhibited so as to extend the model system to encompass studies of granulocyte differentiation.

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Defective erythropoiesis in primary myelofibrosis associated with a chromosome 11 abnormality

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Summary A case of primary myelofibrosis was identified with a previously unreported complex karyotype with two abnormal clones in addition to a proportion of normal cells: 46,XY,− 2,− 11,+ der(2)t(2;11)(q24/31;q13),+ mar and 45,XY,− 2,− 11,+ der(2)t(2;11)(q24/31;q13),+ mar,− 17,del(7q). Study of circulating committed progenitors from this patient consistently showed (1) an absence of erythroid progenitors which is uncommon and (2) greatly increased granulocyte-monocyte progenitors (CFU-GM) which is generally observed in myelofibrosis. Further study showed that peripheral blood mononuclear cells co-cultured with irradiated normal bone marrow stroma generated increased numbers of CFU-GM compared with controls but failed to generate erythroid progenitors, providing evidence for an intrinsic defect in erythropoiesis. Only once previously has the absence of erythroid progenitors in primary myelofibrosis been studied in relation to cytogenetic abnormalities. This case also revealed a complex karyotype which, however, shared with our case a defect on chromosome 11. The identification of two cases of primary myelofibrosis which lack committed erythroid progenitor cells and which show in common a chromosomal defect on chromosome 11 point to the existence of genes on this chromosome which play a key role during erythropoiesis.

Primary myelofibrosis (PMF) is a form of chronic myeloproliferative disease characterised by bone marrow fibrosis, hepatosplenomegaly and the presence of leucoerythroblastic changes and tear drop poikilocytes in the peripheral blood. The finding of G-6-PD isoenzyme restriction in patients' peripheral blood cells, but not fibroblasts and the demonstration of clonal cytogenetic abnormalities in pluripotent haemopoietic progenitor cells support the hypothesis that the primary defect is a clonal expansion of pluripotent haemopoietic progenitor cells associated with reactive marrow fibrosis (Jacobson *et al.*, 1978; Ruutu *et al.*, 1983; Sato *et al.*, 1986; Sugiyama *et al.*, 1989). Reported cytogenetic aberrations have been varied but abnormalities of chromosome 13 have been the most common (Borgstrom *et al.*, 1984; Johnson *et al.*, 1985). PMF is also associated with extramedullary haemopoiesis and increased levels of circulating haemopoietic progenitors including multilineage progenitors (CFU-GEMM), megakaryocyte progenitors (CFU-Mk), erythroid progenitors (BFU-E) and granulocyte-macrophage progenitors (CFU-GM). Many cases have even shown erythropoietin independent erythroid colony formation (Hibbin *et al.*, 1984; Carlo-Stella *et al.*, 1987).

We report on a case of PMF in whom defective erythropoiesis, characterised by the absence of circulating erythroid progenitors and the inability to generate erythroid colonies on irradiated normal marrow stroma, was associated with a complex cytogenetic abnormality. Comparison of the karyotype in this case with that observed in a previously reported case in whom erythropoiesis was defective (Partenen *et al.*, 1982) reveals a common abnormality involving chromosome 11. The significance and possible linkage between defective erythropoiesis and the karyotypic abnormality is discussed.

Case report

A 71 year old man presented with anaemic symptoms, 8 cm hepatomegaly and 12 cm splenomegaly. His Hb was 43 g l^{-1}, Wbc 2.8×10^9 l^{-1} with neutrophils 2.2×10^9 l^{-1} including hypersegmented forms, lymphocytes 0.3×10^9 l^{-1}, monocytes 0.2×10^9 l^{-1}, myelocytes $< 0.1 \times 10^9$ l^{-1}, blasts 0.1×10^9 l^{-1}, normoblasts 1 per 100 w.b.c. and platelet count 95×10^9 l^{-1}. His blood film showed tear drop poikilocytes but infrequent normoblasts which were rarely seen on subsequent blood films. His reticulocyte count was 33×10^9 l^{-1} and both the serum and red cell folate were reduced. Bone marrow aspiration was unsuccessful and a trephine biopsy was hypercellular showing marked granulocytic hyperplasia, absent normoblasts, megakaryocytes of increased size with irregular nuclei and increased reticulin fibrosis, consistent with features in cases reported by Barosi *et al.*, 1983. He was initially supported with folic acid, allopurinol and blood transfusion and was subsequently treated with hydroxyurea and oxymethalone. He remained transfusion dependent but after 7 months developed an increasing leukocyte count with an increasing proportion of blast cells which was refractory to therapy with hydroxyurea. He died 9 months after presentation having followed a course similar to patients reported by Bentley *et al.*, 1977.

Materials and methods

Cells

Peripheral blood mononuclear cells (PBMNC) from the above case collected into preservative free heparin were separated on ficoll-hypaque (Pharmacia, UK) and the interface cells were washed three times in Iscove's modified Dulbecco's medium (IMDM) (Gibco, UK).

Assays for CFU-GM and BFU-E

Fresh PBMNC were assayed for their ability to form colonies in semi-solid medium using a method similar to that described by Ash *et al.*, 1981. For a CFU-GM assay, $2-8 \times 10^4$ PMF PBMNC were seeded in duplicate in a 1.0 ml volume of IMDM containing 1.2% methylcellulose (Sigma), 20% foetal calf serum (FCS, Gibco), 1% deionised bovine serum albumin (Sigma), 5×10^{-5} M 2-mercaptoethanol (Sigma), 5% phytohaemagglutinin stimulated leucocyte conditioned medium (PHA-LCM) and 100 U ml^{-1} penicillin – 50 μg ml^{-1} streptomycin (Gibco). Colonies containing >40 cells were enumerated at day 12. For assessment of BFU-E an identical system containing erythropoietin (Eprex, Cilag UK) at

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3 U ml^{-1} was used, the cells were plated at densities of $0.5-1.0 \times 10^5$ ml^{-1} and the plates read after 14 days. Fresh fractionated human foetal liver cells (Toksoz & Brown, 1984) and normal bone marrow cells were used as positive controls. The assays were performed on three different occasions, firstly at diagnosis and on two other occasions when hydroxyurea therapy had been stopped 96 h previously.

Initiation of haemopoiesis on irradiated stroma from long term bone marrow cultures

Fresh PBMNC were also tested for their ability to initiate haematopoiesis on irradiated stroma from long term bone marrow cultures (LTC). A 10 ml long term bone marrow culture containing 2×10^7 marrow buffy coat cells from a normal marrow donor was established and maintained in medium as previously described (Gartner & Kaplan, 1980). After 30 days nonadherent cells were removed, the adherent cells trypsinised, irradiated with 15 Gray (Co 60 gamma rays, mean energy 1.25 MeV, dose rate 5.3 Gy min^{-1}) and 3 ml of cells at 1×10^5 ml^{-1} reseeded (i.e. at 3×10^4 cm^{-2}) in each of three 35 mm diameter wells of a 6 well tissue culture plate (Nunc). After 5 days incubation, during which a healthy stroma had re-established, 1.5 ml of supernatant was removed from each of the three wells and to each of two wells was added 1.5 ml of LTC medium containing 5.4×10^6 fresh PMF PBMNC. To the remaining well was added 1.5 ml LTC medium alone to act as a negative control. To each of two other empty 35 mm diameter wells was added 5.4×10^6 fresh PMF PBMNC in 3 ml of LTC medium to act as a PMF control. The five cultures were fed weekly by removing half of the supernatant and replacing this with fresh LTC medium. After 5 weeks all nonadherent cells were removed and the cells in the adherent layer harvested by trypsinisation. Nonadherent and adherent cells were pooled, washed and assayed for progenitor cells as described above. The total number of cells harvested from each culture was as follows: PMF PBMNC alone 11×10^5 and 14×10^5; co-cultures of PBMNC and irradiated stroma 15×10^5 and 17×10^5; and irradiated stroma alone 1.4×10^5 cells. The cells in the stroma alone culture were plated in duplicate at a density of 0.7×10^5 ml^{-1} in an erythroid colony assay. Otherwise cells from the other cultures were plated in triplicate in separate assays for CFU-GM and BFU-E at densities ranging from $1.5 \times 10^5 - 2.3 \times 10^5$ ml^{-1}.

Cytogenetic analysis

For analysis of peripheral blood cells, trypsin-Giemsa banded slides were prepared from unstimulated cultures which after 24 h were treated with 0.02 μg ml^{-1} colcemid for either 1 h or 24 h. Metaphases were analysed using standard chromosome criteria. To demonstrate any cytogenetic abnormality in CFU-GM isolated from our patient, pooled GM colonies grown from PBMNC were harvested into IMDM containing 20% FCS and 10% phytohaemagglutinin-leucocyte conditioned medium, and incubated overnight at 37°C in 5% CO_2. Colcemid was added and after 1 h and 24 h cells were harvested and processed as described above.

Results

Cytogenetic analysis

Analysis of 20 metaphases from unstimulated cultures of PBMNC showed three cell lines: one metaphase showed a normal male karyotype; 13 cells showed 46 chromosomes with the loss of one chromosome 2 and one 11, the presence of an abnormal chromosome 2 derived from a 2;11 translocation and an additional unidentified marker chromosome (46,XY,− 2,− 11,+ der(2)t(2;11)(q24/31;q13),+ mar); and six cells showed the above karyotype along with the loss of one chromosome 17 and a deletion of part of the long arm of one chromosome 7 (45,XY,− 2,− 11,+ der(2)t(2;11)(q24/31;q13),+ mar,− 17,del(7q)). Analysis of pooled GM colonies grown from PBMNC showed the third karyotype described above in 8/10 metaphases. The other two metaphases described the same karyotype with the addition of another unidentified marker chromosome, implying some further clonal evolution (Figure 1).

Colony forming ability of PBMNC

On three separate occasions culture of PBMNC revealed greatly increased numbers of circulating CFU-GM with the complete absence of erythroid colonies (Table I). The nature of GM colonies was confirmed following staining of cytocentrifuged preparations of single colony contents. Positive control erythroid colonies were grown from fractionated human foetal liver cells and from bone marrow cells.

Initiation of haemopoiesis on irradiated stroma from LTC

No GM or erythroid colonies were grown from the culture containing irradiated allogeneic normal bone marrow stroma alone and only GM colonies were grown in the other cultures containing PMF PBMNC. Co-cultures containing both irradiated stroma and PMF PBMNC yielded much greater numbers of GM colonies than cultures containing PMF PBMNC alone whether numbers were expressed in terms of total CFU per culture or per 10^5 inoculating cells (Table II). Rowanowsky stained cytospins of cells taken from cultures at the time of clonogenic assay did not show any cells of the erythroid lineage.

Discussion

The main observations from this case study are the demonstration of absent circulating erythroid progenitors in PMF, an uncommon event, and its association with a previously unreported karyotypic abnormality.

Circulating haemopoietic progenitors, particularly CFU-GM, are usually greatly increased in PMF. Studies of circulating erythroid progenitors have shown normal or increased levels in 18/18 cases studied by Carlo-Stella *et al.*, 1987, 17/18 cases studied by Partenen *et al.*, 1982 and 2/2 cases studied by Douer *et al.*, 1983. However, cases of PMF have been reported in which erythroid progenitor cells are absent. In the study of Hibbin and co-workers erythroid progenitors were absent in four splenectomised patients (Hibbin *et al.*, 1984) and Croizat *et al.*, 1983 observed an absence of erythroid progenitors in four patients, two of which had been splenectomised. The absence of circulating erythroid progenitors in PMF patients is not invariably linked to splenectomy since the patient reported in this study and the one identified by Partenen and co-workers with absent BFU-E (Partenen *et al.*, 1982) were not splenectomised. Furthermore, the two larger studies of 18 patients showed normal or increased levels of circulating BFU-E in many splenectomised patients (Partenen *et al.*, 1982; Carlo-Stella *et al.*, 1987).

Many observations support the fact that circulating committed progenitors in PMF largely arise from and circulate from the spleen (Douay *et al.*, 1987). Thus, in the patient reported in this study the failure to detect circulating erythroid progenitors is due to either a defect in the capacity of haemopoietic stem cells to undergo commitment to the erythroid lineage or the ability of committed erythroid progenitors to undergo erythropoiesis or that erythroid progenitors are generated and sequestered in the spleen.

An intrinsic defect in erythropoiesis is the most likely explanation for the following reasons. Douay and co-workers have shown that, in PMF patients, CFU-GM can be maintained in liquid suspension culture in the absence of a substantial stromal layer over a 10-week period and concluded that primitive stem cells circulated in PMF patients (Douay *et al.*, 1987). In our study, PBMNC were co-cultured with normal marrow stroma to maintain circulating primitive stem cells and assess whether they were able to give rise to ery-

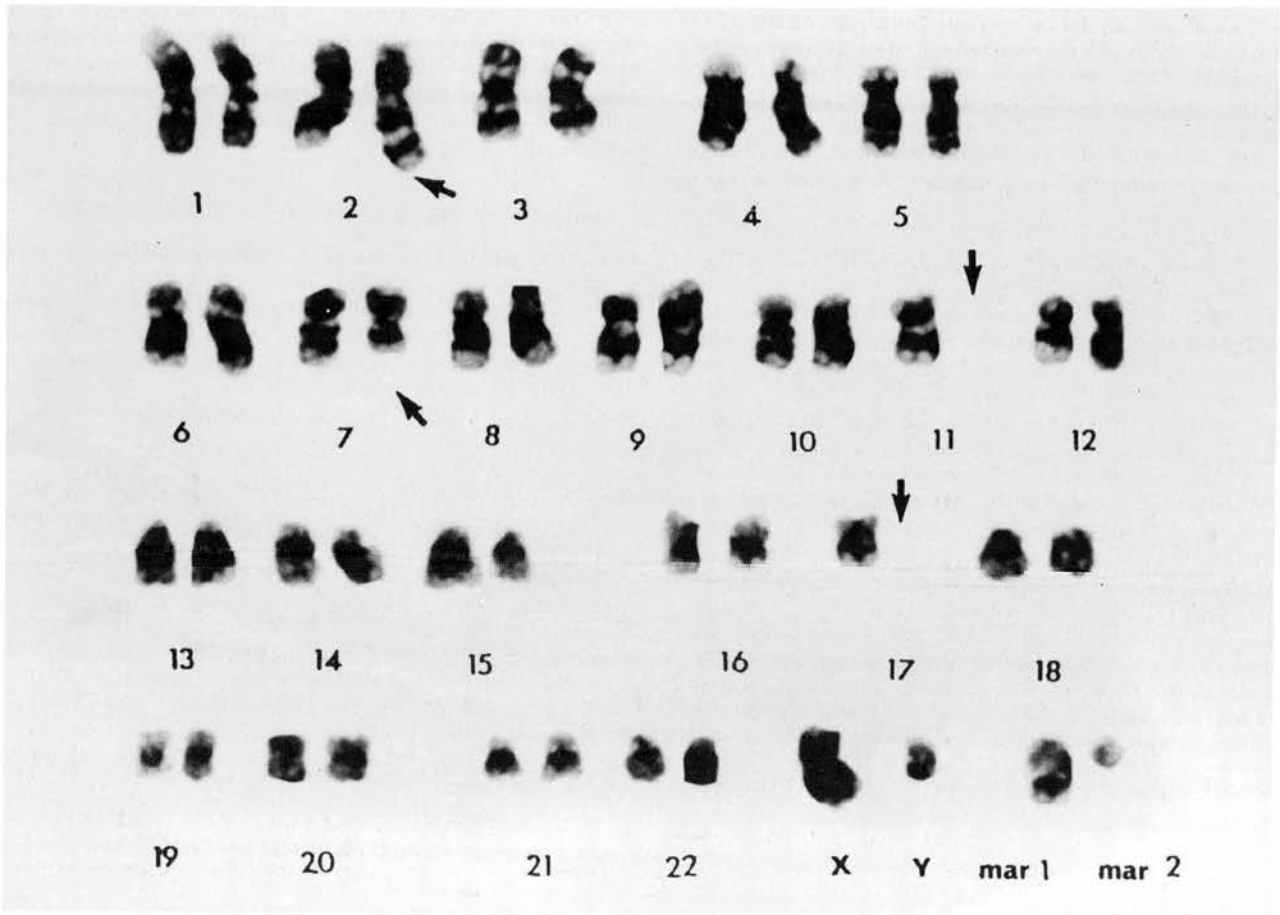

Figure 1 G-banded karyotype from the GM colony culture: 46,XY,− 2,− 11,+ der(2)t(2;11)(q24/31;q13),+ mar1, − 17,del(7q),+ mar2.

Table I Number of colony forming cells grown from PMF PBMNC and normal cells

	PMF PBMNC		*Foetal Liver*		*Bone marrow*	
	CFU ml^{-1} of blood		*CFU per 10^5 cells*			
Time point	*CFU-GM*	*BFU-E/CFU-E*	*CFU-GM*	*BFU-E*	*CFU-GM*	*BFU-E*
1	2,644	0	206	153	33	22
2	7,912	0	244	166	70	28
3	7,605	0	300	90	27	ND

ND = not done.

Table II Mean number (± s.e.m.) of CFU isolated after culture of PMF PBMNC on irradiated bone marrow stroma

	CFU-GM		
Culture condition	*Per 10^5 cells*	*Per total culture*	*BFU-E/CFU-E*
Stroma alone	0	0	0
PMF cells alone	14 (1.1), 11 (1.7)	156 (12), 101 (16)	0
PMF cells + stroma	27 (2.1), 20 (0.7)	405 (31), 348 (11)	0

throid progenitors in a normal and appropriate microenvironment. Erythroid progenitor cells were not generated and the existence of circulating stem cells was supported by the generation of increased numbers of CFU-GM following incubation of PBMNC with marrow stroma as compared with control PMF PBMNC cultured in the absence of stroma. The failure to detect circulating erythroid progenitors for technical reasons can be excluded since the assays were controlled by the demonstration of erythroid progenitors in appropriate numbers from foetal liver and from bone marrow cells.

Of those PMF patients previously studied with absent circulating erythroid progenitors, cytogenetic data is available for only one case which exhibited a complex karyotype – 47,XX,5q − .11q −,− 20,+ mar 1,+ mar 2 (Partenen *et al.*, 1982). However, a common abnormality involving the long arm of chromosome 11 between this and our case is of interest and suggests a causal relationship between an abnormality in the long arm of chromosome 11 and defective erythropoiesis. An abnormality at 11q13 has been reported once previously in a case of PMF, though whether erythropoiesis was defective in this case in unknown (Sessarego *et al.*, 1983). A review of the literature with regard to oncogene associations at the relevant breakpoints in the case

reported in this study reveals that the proto-oncogene SEA (S13 avian erythroblastosis oncogene homolog) maps to the 11q13 region (Williams *et al.*, 1987; Nordenskjold *et al.*, 1989; Hayman *et al.*, 1985). The involvement of this oncogene should be explored at the molecular level.

In conclusion, the association between a distinct cytogenetic abnormality on chromosome 11 and the absence of committed erythryoid progenitor cells in PMF patients is worthy of further study. Cytogenetic analysis of additional patients with an absence of circulating erythroid progenitors might reveal a common chromosome abnormality and point to the location of genes which encode key intrinsic regulators of erythoid lineage development. Furthermore, studies of lesions in the generation of various committed progenitor cells in PMF patients in relation to possible karyotypic abnormalities offers a useful approach to the analysis of the chromosomal location and organisation of genes which control the generation and differentiation of haemopoietic progenitor cells.

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Defective erythropoiesis in myelodysplastic syndromes

We read with interest the report of Williamson *et al* describing uncommon cases of red cell aplasia (RCA) in patients with myelodysplastic syndromes (MDS).[1] The mechanism of RCA in their first three cases was attributed to an intrinsic defect of maturation and proliferation of erythroid precursors occurring as part of the myelodysplastic disorder, whereas in the second three cases a different, and possibly autoimmune aetiology, was suggested. We believe that cytogenetic and molecular study of such unusual cases is important. Most cases of MDS and other malignant haematological disorders are associated with structural chromosomal abnormalities. Cytogenetic study of unusual cases with common features, such as these cases of RCA, might indicate a common chromosomal abnormality which would point to the existence of genes which encode key regulators of erythroid lineage development at or near the junction of the chromosomal aberration. The key regulators of lineage commitment and differentiation in haemopoiesis remain unknown, and an investigative approach through the study of nature's genetic errors might lead to their discovery.

Using such an approach we have recently described a possible association between defective erythropoiesis and an abnormality of chromosome 11.[2] A case of primary myelofibrosis was identified which showed morphological erythroid aplasia and absent circulating erythroid progenitors. The patient had greatly increased numbers of circulating granulocyte-monocyte progenitor cells (CFU-GM). Co-culture of peripheral blood mononuclear cells with irradiated allogeneic normal bone marrow stroma generated increased numbers of CFU-GM compared with controls but failed to generate erythroid progenitors, providing further evidence for an intrinsic defect in erythropoiesis. Our patient exhibited a previously unreported complex karyotype. Only once previously has the absence of erythroid progenitors in primary myelofibrosis been studied in relation to cytogetic abnormalities, and this case also indicated a complex karyotype which shared with our case a defect on chromosome 11. The abnormality in our case was 11q—with the break point at 11q13. A literature review showed that the proto-oncogene SEA (S13 avian erythroblastosis oncogene homolog) maps to the 11q13 region and we intend to study the possible role of this gene at the molecular level.

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Drs Williamson et al comment:
The case cited above is certainly of interest in investigating the pathogenesis of red cell aplasia in myelodysplasia (MDS). The overlap of morphological features between MDS and myelofibrosis is increasingly being recognised,[1] and it would be intriguing to know the consequences at the gene level in this case of 11q- with break point at 11q13.

Our cases all had attempted cytogenetic analysis of marrow aspirate. Four gave a normal karyotype and two failed to yield metaphases. Several of our cases date from the early 1980s and the cytogenetic technique has undoubtedly improved since then. Our rate of clonal cytogenetic abnormality in MDS in the Wessex region is about 35–40% which accords with the national average. A few published series quote clonal abnormality rates of 75% plus, but these may be the result of repeated analysis of large numbers of cells and may be influenced by case selection. In the Wessex region we have never encountered the 11q13 region as being involved in a case of MDS. The 1988 Catalogue of Chromosomal Aberrations in Cancer[2] lists only five reported cases where the 11q13 region has been involved in myelodysplastic/proliferative disorders, often as part of a complex karyotype. A review of the other published cases of MDS and red cell aplasia referenced in our paper shows that all five in which cytogenetics are reported yielded a normal karyotype. Thus although of some interest, it remains to be seen if the 11q13 region has a role in the regulation of erythropoiesis.

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Visceral leishmaniasis in human immunodeficiency virus disease

I read the very interesting article by Curry, Turner, and Lucas.[1] It is important to include visceral leishmaniasis (VL) as an opportunistic protozoan infection in patients infected with HIV, as it is common in endemic areas.[2] Although the authors comment on some of the salient diagnostic and therapeutic features of VL in patients with HIV, the description is perhaps incomplete as it is based on only a few cases. Over the past few years, most cases of VL in HIV infection have been reported from Spain, probably due to a high incidence of both VL and HIV infection and a greater awareness about this association once the first few cases had been described. In 1990 two independent studies described the features of VL in many cases of HIV.[2,3] More recently, our cooperative multicentre study of 40 patients, including most cases that had appeared in Spain up to the beginning of 1990, has updated that experience.[4]

Although in some patients VL can be the consequence of reactivated disease, the finding that 92·5% of the patients were intravenous drug misusers suggested that the disease could be transmitted intravenously (which is an occasional route of transmission in immunocompetents). VL can occur at all stages of HIV infection, but 77% of patients were classified as stage IV with CD4 counts below 4×10^9/l, suggesting that it is commoner in the later stages of HIV infection. Most patients present with a clinical picture of "classic" Kala-azar with fever, hepatosplenomegaly, and pancytopenia, but some are asymptomatic and are diagnosed incidentally. In all patients *Leishmania amastigotes* were demonstrated in the bone marrow smear, and in the liver of 94·5% of the patients who had a biopsy. In four cases *L amastigotes* were found in normal skin, and were also present in skin lesions of a Kaposi's sarcoma in one case. This is not a surprising finding,as *L amastigotes* are found in normal skin in immunocompetent patients with VL. The two most remarkable findings were the absence of leishmanial antibodies, present in only 35·2% of cases, and the chronic relapsing course of the disease. Although 75% of patients had a good initial response to antimony drugs, 42·5% followed a chronic or relapsing course; 40% of patients died due to HIV related causes, and death was only inadvertently related to the relapsing course of the disease.

These findings suggest that VL behaves like other infections in HIV seropositive patients, such as tuberculosis or *Pneumocystis carinii*, showing a good response to initial treatment but persisting as latent chronic disease. Conventional treatment with antimony is not effective in half the patients and other therapeutic approaches are needed. Production of α-interferon (α-INF) and other lymphokines are essential to activate macrophages, but these are defective in both VL and HIV infections. Adjuvant treatment with γ-INF has been effective in animals, in experimental models of human macrophages, and in refractory VL in immunocompetent patients.[5] These results suggest that γ-INF could also be effective in VL in HIV seropositive patients and a therapeutic trial is currently being conducted in Spain.

VL is an opportunistic infection in HIV seropositive patients that is found increasingly often. It must be suspected and precluded in patients presenting with fever, hepatosplenomegaly, and pancytopenia, and even in less ill patients living in or travelling to endemic areas.

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Levels of inositol metabolites within normal myeloid blast cells and changes during their differentiation towards monocytes

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SUMMARY

A homogeneous population of undifferentiated myeloid blast cells was purified from human fetal liver by rosette sedimentation of erythroblasts and macrophages, after coating these cells with monoclonal antibodies, followed by a cell elutriation step. The undifferentiated blast cells were maintained in culture, in a serum-free medium containing 1 mg l^{-1} inositol, by the presence of a high concentration of interleukin-3 (100 U ml^{-1}). This allowed equilibrium labelling of cells with [2-^{3}H]*myo*-inositol and analysis of the concentrations of inositol metabolites. The myeloid blast cells contained high concentrations of an unidentified inositol metabolite, possibly *sn*-glycero-3-phospho-1-inositol (Gro*P*Ins, 22 μM), inositol monophosphate (Ins*P*, 16 μM), an unidentified inositol bisphosphate (InsP_2, 9.4 μM), inositol pentakisphosphate (InsP_5, 37 μM) and inositol hexakisphosphate (InsP_6, 31 μM). These high concentrations are similar to those reported in the promyeloid cell line, HL60. Treatment of the blast cells with 10 nM phorbol myristate acetate (PMA) resulted in rapid differentiation of 48% of the cells towards monocytes. Notable changes in the levels of inositol metabolites included an increase in the putative Gro*P*Ins peak (to 73 μM) and decreases in the concentrations of InsP_4 (from 4 μM to 1 μM) and InsP_5 (to 21 μM). These changes in response to PMA, with the exception of the rise in the putative Gro*P*Ins, are similar to those reported in HL60 cells undergoing monocyte differentiation. These observations suggest that the abundant inositol polyphosphates may have an as yet unknown role in myeloid differentiation.

1. INTRODUCTION

The breakdown of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) following receptor activation by an agonist is now well established as a component of an important signalling system in cells (Berridge & Irvine 1989; Downes & Macphee 1990). Cleavage of PtdIns(4,5)P_2 leads to the production of two distinct messengers, inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and diacylglycerol (DAG). Ins(1,4,5)P_3 generation leads to the release of Ca^{2+} from intracellular stores (Rasmussen & Rasmussen 1990; Berridge 1990) and DAG activates protein kinase C, leading to phosphorylation of a number of cellular proteins (Nishizuka 1988; Parker *et al.* 1990). However, these inositol metabolites involved in cellular signalling represent only a small fraction of the complex pattern of inositol metabolites present in cells. In particular, cells contain high concentrations of the highly phosphorylated inositol metabolites, inositol pentakisphosphate (InsP_5) and inositol hexakisphosphate (Heslop *et al.* 1985; Szwergold *et al.* 1987; Jackson *et al.* 1987).

Our recent studies of the inositol phosphates present within the HL60 cell line have revealed an extremely complex pattern of inositol metabolites, with concentrations of InsP_5 and InsP_6 of about 25 μM and 60 μM, respectively. Furthermore, the concentration of InsP_5 changes dramatically as HL60 cells differentiate towards either neutrophils or monocytes. During neutrophil differentiation there was a threefold increase in the intracellular concentration of InsP_5 and, in contrast, there was a 90% decrease in the level of InsP_5 as HL60 cells differentiated toward monocytes (French *et al.* 1991). These studies of HL60 cells have suggested that the abundant inositol polyphosphates may have some, as yet unknown, role in myeloid differentiation. It is, therefore, important to investigate whether high levels of the highly phosphorylated species are a feature of either immature myeloid cells or leukaemic cell lines, and also whether the observed changes in levels occur in normal myeloid cells as they undergo differentiation.

To approach these questions, a morphologically homogeneous population of myeloid blast cells has been purified from human fetal liver. Our previous studies have shown that fetal liver is a rich source of

myeloid progenitor cells (Toksoz & Brown 1984). A relatively homogeneous population of myeloid blast cells has been obtained from fetal liver by using a negative selection procedure, so as not to interfere with subsequent physiological studies (Bunce *et al.* 1990). These blast cells differentiate spontaneously in culture towards neutrophils and monocytes. However, when treated with phorbol myristate acetate (PMA) 62% of the blast cells undergo rapid differentiation towards monocytes, and the spontaneous differentiation of the remaining blast cells towards neutrophils is inhibited (Bunce *et al.* 1990). In this study the blast cell population has been further fractionated by cell elutriation to obtain a population of promyeloid cells which is more uniform as regards their differentiation status. These have been equilibrium-labelled with [2-^{3}H]*myo*-inositol, in a serum-free medium containing 1 mg l^{-1} inositol and a high concentration of interleukin-3 (IL-3) and then treated with PMA to induce rapid differentiation towards monocytes. The concentrations of inositol metabolites within the undifferentiated blast cells and the changes which occur during their monocyte differentiation have been studied in detail.

2. MATERIALS AND METHODS

(*a*) *Purification of blast cells*

Abortuses from prostaglandin-induced termination of mid-trimester pregnancies were obtained from two local hospitals. All handling of fetal tissue was done in agreement with the Polkinghorne Report, and was supervised within the University of Birmingham by a central co-ordinator. Under these guidelines, maternal consent for the use of fetal material for research purposes was obtained by a neutral third party and the identity of patients and source of fetal material was unknown to the investigators. This research was done under special license from the Department of Health, and in addition was approved by the local hospital ethical committee.

A single cell suspension was prepared from whole human fetal livers (16–18 weeks gestation) as follows. Tissue was gently teased into small fragments, passed through a 19 *g* needle and incubated for 40 min at 37 °C in RPMI 1640 medium (Gibco, Paisley, U.K.) containing collagenase H (0.5 mg ml^{-1}, Boehringer Mannheim, Lewes, U.K.), dispase (1.0 mg ml^{-1}, Boehringer Mannheim) and hyaluronidase (0.5 mg ml^{-1}, Sigma, Poole, U.K.). Enzymic digestion of the tissue fragments was inhibited by the addition of an equal volume of cold RPMI 1640 medium supplemented with 10% (by volume) fetal calf serum (FCS) (GIBCO) and the cells were recovered by centrifugation at 500 *g* for 10 min. The preparation of light-density cells from the resulting single cell suspension and the removal of erythroblasts and macrophages from this population of cells have been described in detail previously (Bunce *et al.* 1990). Briefly, light-density cells were purified by fractionation of the above cell preparation on Ficoll-Hypaque (Pharmacia, Milton Keynes, U.K.; (Boyum 1968)), which was done twice. The light density cells were coated with monoclonal antibodies against glycophorin C (Ret 40F, Dr D. Y. Mason, (Gatter *et al.* 1988)) and a monocyte cell surface antigen (61D3, (Nunez *et al.* 1982)) and the cells were rosetted with sheep erythrocytes coated with anti-mouse immunoglobulin (Ling *et al.* 1977). The rosette-positive cells and excess sheep erythrocytes were removed by fractionation on Ficoll-Hypaque and the interface cells washed twice in RPMI 1640 supplemented with 2% (by volume) FCS before being fractionated by counterflow cell elutriation (CCE). CCE was done at 10 °C and at 1950 r.p.m. using a Beckman J-6M centrifuge with a JE-6B elutriator rotor fitted with a standard chamber (Beckman Instruments, Glenrothes, U.K.). Cells were loaded at a flow rate of 4.7 ml min^{-1} in phosphate-buffered saline containing 2% FCS, and eight 100 ml fractions were collected at flow rates of (in ml min^{-1}): 4.7, 9.0, 13.3, 17.8, 22.0, 26.3, 30.4 and 34.5. A final 'rotor off' fraction was collected as the centrifuge came to rest at the end of the procedure. For culture experiments, cells which eluted at 13.3 and 17.8 ml min^{-1} were combined and washed once (220 *g*) in RPMI 1640 medium containing 1 mg l^{-1} inositol and supplemented with the serum replacement ITS+ (see below).

(*b*) *Culture of cells and induction of differentiation*

After washing, the elutriated blast cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2, by using a serum-free medium consisting of inositol-free RPMI 1640 (Northumbria Biologicals Ltd, Northumberland, U.K.) supplemented with 1 mg l^{-1} inositol, ITS+ (6.3 mg l^{-1} insulin, 6.3 mg l^{-1} transferrin, 6.3 μg l^{-1} selenous acid, 1.3 g l^{-1} bovine serum albumin and 5.4 mg l^{-1} linoleic acid (Flow Laboratories, Rickmansworth, U.K.)), 20 mm glutamine (GIBCO), antibiotics (100 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin, GIBCO) and 100 U ml^{-1} recombinant human interleukin-3 (IL-3) (Genzyme, Koch Light, Hatfield, U.K.). 1 ml cultures were seeded with 5×10^5 cells in 24 well plates (Nunc, Paisley, U.K.). For the study of levels of inositol metabolites and their changes during differentiation, blast cells were labelled to isotopic equilibrium with 2 μCi ml^{-1} [^{3}H]inositol† (TRK 883, Amersham International, Amersham, U.K.) for five days before the addition of 10 nM PMA (Sigma). [^{3}H]inositol was maintained in the medium at the same specific radioactivity during differentiation. Differentiation was assessed by staining of cytospin preparations with May–Grunwald–Giemsa and for monocyte specific esterase using α-naphthyl acetate as the substrate (Yam *et al.* 1971). Staining for CD10 (J5, Coulter, Luton, U.K.), CD34 (ICH_3, Professor R. Levinsky) and CD15 (AGF4.48 (Fisher *et al.* 1982)) positivity was by the indirect immunofluorescence technique, either in suspension (CD10 and CD34) or on cytocentrifuged preparations (CD15).

(*c*) *Analysis of inositol lipids and phosphates*

The acidic extraction of lipids and inositol phosphates and high performance liquid chromatography (HPLC) separation of inositol phosphates have been described in detail previously (French *et al.* 1991). The radioactivity in each HPLC peak was converted to an intracellular concentration, averaged throughout the cell, by using values for the specific activity of [^{3}H]inositol in the growth medium and for the cell volume, calculated from the mean cell diameter (assuming the cells to be perfect spheres). The concentration of inositol in the culture medium was measured using a chemiluminescence assay (Gudermann & Cooper 1986).

3. RESULTS

(*a*) *Purification of myeloid blast cells*

As described previously, the cell population obtained from fetal liver mononuclear cells after rosette depletion

† 1 Ci = 3.7×10^{10} Bq.

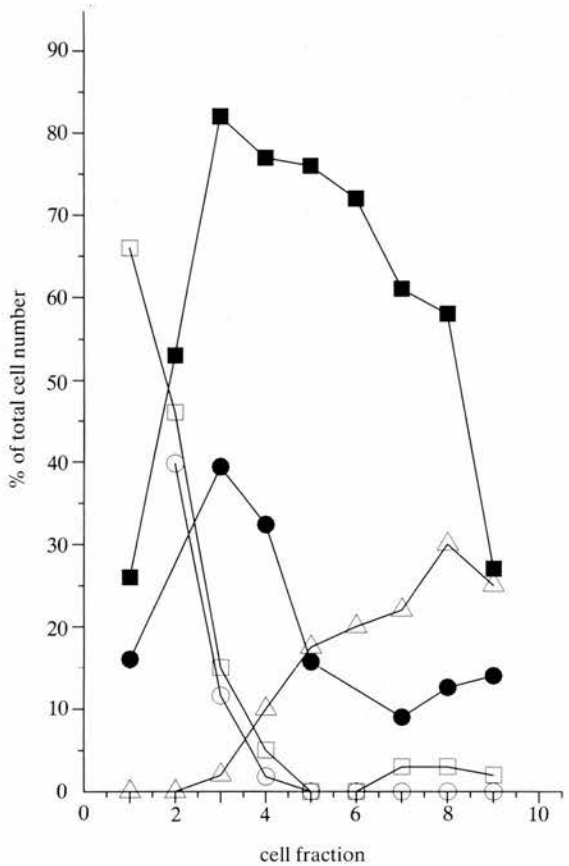

Figure 1. Elutriation profile of fetal liver blast cells. Blast cells were enriched by negative selection as described in §2 before fractionation by counterflow cell elutriation. Fractions were collected at the incremental flow rates defined in §2. Cytospun preparations of the cells were analysed by Romanovsky staining to reveal the percentages of lymphocytes (open squares), blast cells (closed squares), and promonocytes (open triangles). Cells were stained in suspension by indirect immunofluorescence to identify cells expressing the CD10 (open circles) and CD34 (closed circles) antigens.

of erythroblasts and monocytes consisted of 73% undifferentiated blast cells, cells at the promyelocyte to neutrophil stages of differentiation (9%), lymphocytes (4%), and unidentified cells (11%). The blast cells exhibited variation in cell size and both nuclear and cytoplasmic characteristics (Bunce *et al.* 1990). These cells were therefore further fractionated by the use of a cell elutriator (figure 1). The first two fractions contained cells with a lymphoid appearance, which represented 46–66% of the cells in these fractions. Immunofluorescence studies revealed that these cells express the CD10 antigen and are therefore most likely to be B-cell progenitors. Undifferentiated blast cells were eluted predominantly in fractions 3–7, obtained at flow rates of 13.4–30.4 ml min^{-1}. However, as shown in figure 1, beyond fraction 4 there was increasing contamination of the undifferentiated blast cells with cells that had the clear morphological appearance of promonocytes. These cells, identified in Romanovsky-stained preparations, had the distinguishing characteristics of increased size with increased quantities of cytoplasm that was less basophilic, vacuolated and had irregular edges. The nuclei of these cells were eccentrically placed, reniform in shape and contained nucleoli. To achieve optimal separation of undifferentiated blast cells from promonocytes and lymphoid progenitor cells, cells within fractions 3 and 4 (obtained at flow rates of 13.3 ml min^{-1} and 17.8 ml min^{-1}) were pooled for use in experiments. In five experiments, the mean yield of elutriated blast cells was 2.1×10^6 ($\pm 0.7 \times 10^6$ (s.e.)). Analysis of Romanovsky-stained preparations of the purified cells used in experiments showed that they were mostly blasts (73.9%), lymphoid cells (14.3%) and promonocytes and cells at the promyelocyte to neutrophil stages of maturation (11.8%). Comparison of the morphology of the blast cell populations before and after cell elutriation revealed that the elutriated blast cells were more uniform in size and nuclear characteristics. The nuclear outline of the elutriated blast cells was more regular, and the chromatin pattern contained multiple nucleoli. The cytoplasm of these cells was scant to moderate in quantity and basophilic. Cytochemical staining showed that the blast cells did not express chloroacetate esterase or monocyte-specific esterase. They were not stained by procedures for either Sudan black or periodic acid Schiff positivity. Thus the blast cells lacked characteristics of the precursors of either neutrophils or monocytes. A considerable percentage of the blast cells (32–40%) expressed the CD34 antigen, which is present on haemopoietic progenitor cells and some myeloid cells (Peschel & Köller 1989).

(*b*) *Maintenance and differentiation of blast cells in culture*

When the purified blast cells were cultured in serum-free medium containing 1 mg l^{-1} inositol and ITS+ there was rapid loss of cell viability such that by day 2 the cells had declined in number from 5×10^5 ml^{-1} (figure 2*a*). The addition of IL-3 to the culture medium, at a concentration of 100 U ml^{-1}, resulted in a small initial decline in cell number at day 2 (to 2.4×10^5) followed by a gradual increase to 5.4×10^5 ml^{-1} at day 5 and 6.4×10^5 at day 7. The nature of the cells that had increased in number was assessed by staining of cytospin preparations (figure 2*b*, table 1). After five days of culture in medium containing IL-3, the cell population still consisted mostly of undifferentiated myeloid cells (85%). By this time, cells with the morphological appearance of lymphocytes had died and they now represented less than 1% of the cell population. The remaining cells were at the promyelocyte to neutrophil stages of differentiation (2%), promonocytes (7.5%) and cells that could not be readily identified (4.6%). Although the morphology of the blast cells changed slightly over five days (predominantly an increased cytoplasm to nuclear ratio) they exhibited no clear evidence of differentiation towards either monocytes or neutrophils. Furthermore, the low rate at which cells spontaneously differentiated in the cultures was confirmed by the small percentages of cells which expressed CD15, an antigen which appears at the promyelocyte stage of neutrophil differentiation (2.0–5.5%) or

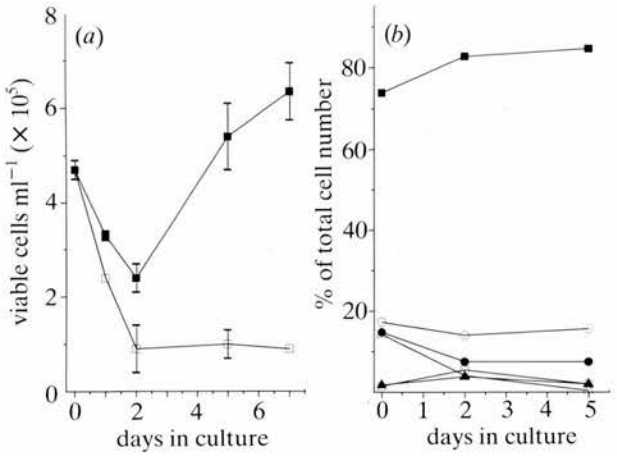

Figure 2. Maintenance of fetal liver blast cells in IL3. (*a*) Elutriated blast cells were cultured in serum-free medium containing no IL3 (open squares) or 100 U ml^{-1} IL3 (closed squares). Cultures were harvested at 0, 1, 2, 5 and 7 days, and the numbers of viable cells were assessed by phase contrast microscopy. (*b*) Cytospun preparations of cells cultured in the presence of IL3 were Romanovsky stained to determine the percentages of blast cells (closed squares), lymphocytes (open triangles), promyelocytes → neutrophils (closed triangles) and promonocytes–monocytes (closed circles). Cytospun preparations were also stained to identify cells which express CD15, an antigen which appears at the promyelocyte stage of neutrophil differentiation (open squares) or α-naphthyl acetate esterase positive cells (open circles).

Table 1. *Differentiation of normal blast cells towards monocytes in response to PMA*

(Cells were cultured for five days in 100 U ml^{-1} IL3[a] before adding 10 nM PMA for a further 24 h[b]. Data are mean ± s.e. of four experiments.)

	percentage of cells	
characteristic	−PMA[a]	+PMA[b]
differential:		
normoblasts	< 1	< 1
blasts	85 ± 1.7	47 ± 8.0
promonocytes	7.5 ± 1.6	48 ± 8.0
monocytes	< 1	< 1
promyelocytes → myelocytes	2.0 ± 0.7	< 1
granulocytes	< 1	< 1
lymphocytes	< 1	< 1
others	4.6 ± 1.0	4.8 ± 1.5
α-naphthyl acetate esterase	15.6 ± 3.6	19.6 ± 3.0

monocyte associated α-naphthyl acetate esterase (15.6–17.3 %) (figure 2).

The addition of PMA to cultures of blast cells which had been grown for five days in medium containing IL-3, and labelled with [^{3}H]inositol, resulted in rapid differentiation of the blast cells towards monocytes. The appearance of the cells in cultures treated with PMA for 24 h had changed; they had become both adherent and pleomorphic. The percentage of cells which had undergone differentiation towards monocytes was assessed by the number of promonocytes and

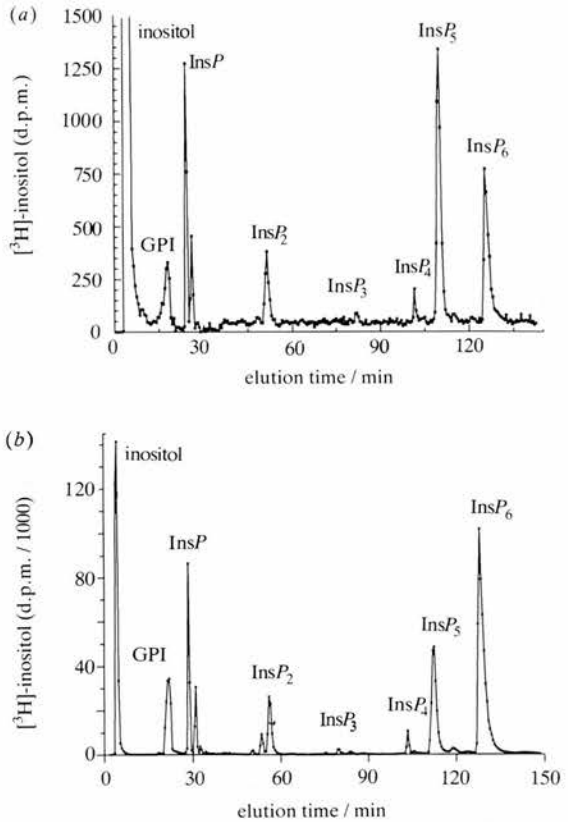

Figure 3. HPLC separation of inositol phosphates from (*a*) fetal liver blast cells and (*b*) HL60 cells. Typical HPLC traces of inositol metabolites extracted from 1×10^6 elutriated blast cells and 1×10^7 HL60 cells (first shown in French *et al.* 1991) are shown. In each case peaks corresponding to inositol (Ins), putative Gro*P*Ins (GPI) and inositol phosphates (Ins*P* → InsP_6) are highlighted.

monocytes in Romanowsky-stained preparations. As shown in table 1, after 24 h of exposure to PMA, 48 % of the cells were classed as promonocytes and monocytes, compared with 7.5 % of cells immediately before PMA treatment. The percentage of cells expressing α-naphthyl esterase had risen only slightly, from 16 % to 20 %. This small rise in α-naphthyl acetate esterase positively after 24 h exposure to PMA is consistent with our previous studies of normal blast cells, in which a significant increase in the percentage of positive cells was not seen until the second day of treatment with PMA (Bunce *et al.* 1990). The above changes are consistent with cells having undergone differentiation towards monocytes.

(*c*) *Levels of inositol metabolites in blast cells and changes during their differentiation*

Analysis of aqueous extracts obtained from undifferentiated blast cells revealed a complex pattern of inositol metabolites. Figure 3 compares the pattern of water-soluble inositol metabolites within undifferentiated blast cells, as revealed by HPLC, with that obtained for exponentially grown HL60 cells in a previous study (French *et al.* 1991). The myeloid blast cells contained a much higher free inositol concentration than HL60 cells. Growing HL60 cells contained approximately

40 μM free inositol, averaged throughout the cell volume (French *et al.* 1991), whereas the blasts contained approximately 2.6 mM (table 2). In contrast to the large differences in inositol content, the relative levels of the inositol phosphates seen in the undifferentiated blasts cells and growing HL60 cells were similar. However, the blast cells contained a higher concentration of InsP_5 than InsP_6, whereas in growing HL60 cells this ratio was reversed. This difference in the relative concentrations of InsP_5 and InsP_6 is unlikely to be related to the leukaemic character of HL60 cells, as another leukaemic myeloid cell line, KG1a, contains levels of the InsP_5 higher than InsP_6 (C. M. Bunce & P. J. French; unpublished observation).

As shown previously in HL60 cells, the undifferentiated blast cells contained remarkably high concentrations of a number of inositol metabolites. These included a compound which eluted between inositol and inositol monophosphates on HPLC (probably Gro*P*Ins; 22 μM), Ins1*P* or Ins3*P* and Ins2*P* (16 μM), InsP_5 (37 μM) and InsP_6 (31 μM). Lower concentrations were observed of an inositol bisphosphate which co-eluted with Ins(1,4) P_2, an inositol trisphosphate which co-eluted with Ins(1,4,5) P_3 and an inositol tetrakisphosphate which eluted close to Ins(1,3,4,5) P_4 (Table 2). Co-elution of the peaks on HPLC with authentic standards only provides an indication of their possible identities, as other inositol polyphosphate isomers may also elute within some of these peaks.

The concentrations of certain inositol metabolites within the myeloid blast cells changed in response to a 24 h exposure to PMA. Significant decreases were observed in the levels of both InsP_4 and InsP_5. The level of InsP_4 decreased by about 75% from 4.2 μM to 1.1 μM, and the level of InsP_5 decreased by about 50%, from 37 μM to 21 μM. In contrast, there was a marked increase in the level of the putative Gro*P*Ins from 22 μM to 73 μM. No significant changes were observed in the levels of free inositol, Ins*P*, InsP_2, InsP_3 or InsP_6.

Table 2. *Intracellular concentrations of inositol and inositol phosphates in normal blast cells*

(Cells were cultured for five days in serum-free medium containing 100 U ml^{-1} IL3 and [^{3}H]inositol. On day five, PMA was added to a final concentration of 10 nM. Control[a] and treated[b] cells were harvested 24 h later. Data are means ± s.e. of values obtained from four experiments, except for InsP_3 which represents one determination only.)

	concentration (μM)	
inositol derivative	−PMA[a]	+PMA[b]
inositol	2610 ± 1200	2030 ± 761
Gro*P*Ins	22.4 ± 2.1	73.2 ± 17.0
Ins1/3*P* & Ins2*P*	16.2 ± 2.1	14.8 ± 1.4
InsP_2	9.4 ± 4.6	12.9 ± 1.2
InsP_3	0.8	1.0
InsP_4	4.2 ± 0.7	1.1 ± 0.4
InsP_5	37.2 ± 2.7	20.7 ± 3.5
InsP_6	30.5 ± 5.1	27.2 ± 3.5

4. DISCUSSION

A population of myeloid blast cells with a homogenous primitive morphology has been purified from human fetal liver and maintained in serum-free medium containing high levels of IL3. The potential of IL3 to support the blast cells in culture is analogous to the maintenance of the undifferentiated status of murine IL3 dependent cell lines. (Heyworth *et al.* 1990). Labelling of blast cells under these culture conditions, in medium with a known specific activity of [^{3}H]inositol, and measurement of the mean cell volume has allowed us to determine the concentrations of inositol metabolites within these cells. The levels of several inositol phosphates within undifferentiated myeloid blast cells were similar to those observed within the promyeloid cell line HL60 (French *et al.* 1991). The concentrations of Ins*P*, InsP_2, InsP_4 and InsP_5 observed in blast cells and HL60 cells, respectively, were as follows: Ins*P*, 16 μM against 24 μM; InsP_2, 9 μM against 14 μM; InsP_4, 4.2 μM against 4.4 μM and InsP_5, 37 μM against 27 μM. Other compounds display differences in concentration between the two cell populations. HL60 cells contained more InsP_6 than the fetal liver blast cells (63 μM against 31 μM) and less Gro*P*Ins (11 μM against 22 μM). A particularly striking difference between the two cell populations is the concentration of free intracellular inositol. Within the blast cells the concentration of inositol was 2.6 mM, which is much higher than the 39 μM observed within HL60 cells. This may in part be accounted for by the fact that the blast cells were obtained from fetal tissue, which has a high extracellular concentration of inositol in the surrounding fluids (125 μM, (Lewin *et al.* 1978; Quirk & Bleasdale 1983)). In contrast, the HL60 cells had been maintained long term in medium containing low amounts of inositol (5 μM). The similar levels of higher inositol phosphates found in cells with very different-sized pools of free intracellular inositol suggests that cells can actively control their levels of highly phosphorylated derivates even when the level of intracellular inositol is restricted.

The changes described previously in inositol phosphates during HL60 differentiation towards monocytes included rapid and sequential declines in InsP_4 and InsP_5, and a slower fall in InsP_6 which was not significant until 48 h (French *et al.* 1991). In HL60 cells treated for 24 h with PMA there was a 75% decrease in the level of InsP_4, an 80% decrease in the level of InsP_5 and no change in the level of InsP_6. Similar changes in the levels of these three compounds were observed at 24 h when the fetal blast cells differentiated towards monocytes in response to PMA: the level of InsP_4 had declined by 75% and that of InsP_5 by 50%, whereas InsP_6 remained unchanged. It is possible that the change in the concentration of InsP_4 seen in both cell types is related to activation, by protein kinase C, of the 5-phosphatase which degrades Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4, which is likely to be a major component of the InsP_4 peak. However, in the HL60 studies no depletion of Ins(1,4,5)P_3 was observed in response to PMA, which contradicts this possible explanation for the depletion of InsP_4. The decreases in the levels of

$InsP_4$ and $InsP_5$ which occurred in both normal myeloid blast cells and HL60 cells undergoing monocyte differentiation may be a key feature of cell maturation along this pathway. It will, therefore, be of particular interest to determine whether similar decreases in the amounts of $InsP_4$ and $InsP_5$ occur in the normal blast cells as they undergo differentiation in response to M-CSF. However, as yet we cannot undertake an analysis of the changes in inositol metabolism in this situation. This is because the blast cells committed to neutrophil and monocyte differentiation cannot, at present, be separated, and addition of M-CSF to the mixed blast cell population does not result in sufficient promotion of monocyte differentiation such that this exceeds the concomitant, spontaneous granulocyte differentiation. Thus, appropriate analysis of inositol metabolites cannot as yet be undertaken.

As the blast cells underwent differentiation towards monocytes there was an increase in the concentration of the putative Gro*P*Ins peak (from 22 μM to 73 μM). This was not observed as HL60 cells differentiated towards monocytes, but there was a fourfold rise in the level of this peak during HL60 neutrophil differentiation (French *et al.* 1991). Preliminary studies in HL60 cells have shown that mild periodate treatment of this compound followed by reduction by dimethylhydrazine yields predominantly Ins(1/3) *P*, suggesting that most of this material is *bona fide* Gro*P*Ins (data not shown). If this peak is indeed Gro*P*Ins, then its accumulation could be a reflection of PtdIns deacylation by phospholipase A_2 (PLA_2) and a lysophospholipase (as suggested in Alonso & Santos 1990). An important difference between the cell populations might therefore lie in the control of PLA_2.

In summary, normal myeloid blast cells and HL60 promyelocytic cells contain a similar complement and levels of inositol phosphates. These two cell populations also show similar changes in inositol metabolism as they onset differentiation towards monocytes in response to treatment with PMA. These data support the suggestion, from our studies of HL60 cells (French *et al.* 1991), that the inositol polyphosphates have some role in the process of myeloid cell differentiation. In future studies, it will be important to undertake analysis of changes in the levels of inositol metabolites within precursor cells as they undergo differentiation in response to appropriate physiological factors.

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